# INTERACTIONS AND REACTIONS BETWEEN DIFFERENT TYPES OF PROTEINS AND THEIR IMPORTANCE IN WHEAT-BASED MODEL AND NOODLE SYSTEMS

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# Voorwoord

Beste lezer,

U opent deze doctoraatsthesis uit interesse, waarvoor dank! U staat op het punt om een klein wetenschappelijk avontuur te ontdekken waar ik vier jaar aan heb gewerkt. Misschien kijkt u er tegenop om alle bladzijden één voor één door te nemen, maar ik hoop dat u de tijd neemt om in deze thesis te kijken, te snuisteren, te lezen en/of te leren. Ik hoop oprecht dat dit manuscript een even grote verrijking zal zijn voor u als het was/is voor mij. Ja, ik mag dit laatste in de verleden tijd schrijven, want dit voorwoord is voor u het begin, maar voor mij het einde van dit 'boekje'. Desalniettemin is de tegenwoordige tijd beter toepasbaar gezien de verworven kennis, ervaring, inzicht, diploma en herinneringen blijvend zijn. Dit doctoraatswerk is echter niet alleen tot stand gekomen. Vele mensen hebben mij de voorbije jaren geholpen, gesteund en kansen gegeven. Tijd dus om iedereen eens extra in de bloemetjes te zetten!

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Marlies
Oktober 2016

# Samenvatting

Proteïnen bepalen de structurele eigenschappen van veel tarwegebaseerde levensmiddelen. De meest voorkomende proteïnen in tarwe zijn glutenproteïnen. De term glutenproteïnen wordt gebruikt voor monomeer gliadine en polymeer glutenine. Samen vormen zij een netwerk wanneer tarwebloem gemengd wordt met water. Glutenproteïnen polymeriseren tijdens verhitting voornamelijk via het aanleggen van disulfidebindingen. Op die manier dragen ze bij tot onder andere visco-elastische eigenschappen van tarwegebaseerde degen, de kruimstructuur van brood en de kookeigenschappen van pasta of noedels.

In verschillende tarwegebaseerde levensmiddelen zoals cakes, (pannen)koeken, wafels, brood, tortilla's, pasta en noedels, komen tarweproteïnen samen voor met globulaire proteïnen van ei-, soja- of melk. De literatuur geeft voorbeelden van de beïnvloeding van het gedrag van verschillende types van proteïnen op elkaar en hanteert daarvoor de term "co-proteïne effecten". Deze kunnen synergistisch of antagonistisch zijn. Wanneer het bijvoorbeeld over polymerisatiegedrag gaat, dan treedt een synergetisch (of antagonistisch) effect op wanneer de polymerisatie meer (of minder) intens optreedt in het mengsel van twee types proteïnen dan kan voorspeld worden op basis van de waarnemingen voor de individuele proteïnen. Hoewel interacties en reacties tussen verschillende typen proteïnen het gedrag van voedingssystemen beïnvloeden, is het voorkomen van co-proteïne effecten niet goed begrepen. Nochtans kan meer fundamentele kennis over de invloed van ei-, soja- en weiproteïnen op proteïnenetwerkvorming in tarwedeeg en tijdens de verdere verwerking de basis vormen voor de ontwikkeling van levensmiddelen met verbeterde eigenschappen.

Dit doctoraatswerk had tot doel interacties en reacties tussen verschillende typen proteïnen en de invloed hiervan op tarwegebaseerde levensmiddelen te bestuderen.

In een eerste deel werd de impact van verschillende co-solventen in extractiemedia en eluenten bestudeerd op andere dan grootte gerelateerde effecten in gelpermeatiechromatografie. De meeste analysetechnieken berusten op het oplosbaar zijn van proteïnen. Terwijl gluten niet oplosbaar zijn in water of zoutoplossingen zijn ei-, soja- en weiproteïnen dat wel. Co-solventen zijn nodig om glutenproteïnen in oplossing te brengen, maar kunnen met de matrix van de scheidingskolom interageren en zo de scheiding en dus de schijnbare moleculaire gewichtsverdeling beïnvloeden. Deze problemen werden niet waargenomen wanneer als extractie- en elutiemedium een natriumdodecylsulfaat (SDS)-bevattende buffer werd gebruikt. Een methode werd geoptimaliseerd om hitte-geïnduceerde covalente netwerkvorming van verschillende typen proteïnen te bestuderen aan de hand van het verlies in extraheerbaarheid in SDS bevattende buffer en veranderingen in moleculaire gewichtsverdeling.

In een tweede deel werd hitte-geïnduceerde polymerisatie (bij 100 °C) onderzocht in modelsystemen voor geïsoleerde tarwe-, ei-, soja- en weifracties zowel in water als in waterige ethanoloplossingen. Meer proteïnen werden opgenomen in het proteïnenetwerk in water dan in waterige ethanoloplossingen. De resultaten van de geïsoleerde proteïnen werden vergeleken met deze van hun mengsels met gluten. In sommige gevallen werd een synergistisch co-proteïne effect waargenomen, namelijk wanneer proteïnen meer polymeriseerden in mengsels met gluten dan verwacht op basis van de gewogen gemiddeldes van de resultaten van de geïsoleerde proteïnen. Fasescheiding verhinderde het optreden van synergistische co-proteïne effecten niet. Zowel in water als in waterige ethanoloplossingen beïnvloeden verschillende typen proteïnen elkaars denaturatie en polymerisatie. Een model werd ontwikkeld om co-proteïne effecten tussen globulaire- en glutenproteïnen tijdens verhitting bij 100 °C in water te voorspellen. De sleuteleigenschappen die co-proteïne effecten bepalen in mengsels met gluten zijn de hoeveelheid hydrofobe sites en toegankelijke sulfhydryl-groepen van ontvouwde globulaire proteïnen.

In een derde deel werden bovenstaande bevindingen getoetst door noedels, een reëel levensmiddelensysteem, te bestuderen. Niet-covalente interacties domineerden de eigenschappen van verse noedels terwijl covalente en waterstofbindingen een voorname rol speelden voor gekookte noedels. Waarschijnlijk beïnvloeden ionische en hydrofobe interacties de eigenschappen van noedels door het verhinderen van covalente interacties tijdens verhitting. Toevoeging van volledig ei verbeterde de eigenschappen van tarwegebaseerde noedels meer dan toevoeging van eiwit of eigeel afzonderlijk. Proteïnen met een hoog aantal toegankelijke sulfhydryl-groepen konden snel de

vorming van disulfidebindingen initiëren. Dit verlaagde de flexibiliteit van het proteïnenetwerk zodat het minder weerstand kon bieden tegen het zwellen van zetmeel tijdens koken. Desalniettemin resulteerde onvoldoende polymerisatie tijdens koken eveneens in een zwakke noedelstructuur zodat veel materiaal vrijkwam in het kookwater.

We kunnen besluiten dat verschillende typen proteïnen elkaars netwerkvorming beïnvloeden via niet-covalente interacties en covalente bindingen. Hoge gehaltes aan toegankelijke sulfhydryl- en hydrofobe groepen in globulaire proteïnen verhogen de incorporatie van glutenproteïnen in een proteïnenetwerk. Nochtans kan een te snelle en overmatige incorporatie van proteïnen in het netwerk de kwaliteit van noedels reduceren. Een optimaal proteïnenetwerk is cruciaal voor een superieure noedelkwaliteit. Toevoeging van volledig ei, soja glycinine of runderserumalbumine verbetert de eigenschappen van gekookte noedels.

# Summary

Proteins impact the structural quality of wheat-based food products. The main proteins of wheat are gluten proteins. These consist of monomeric gliadin and polymeric glutenin. They form a network when wheat flour is mixed with water and polymerize even further upon heating, mainly through disulfide bond formation. Gluten network formation is responsible for *inter alia* the visco-elastic properties of wheat-based dough, the crumb structure of bread and the cooking properties of pasta or noodles.

In many wheat-based food products including some cake, cookie, pancake, waffle, bread, tortilla, pasta and noodle systems wheat proteins coexist with globular proteins from egg, soy or milk. Under specific conditions, different proteins interact or react differently than similar proteins. Literature provides examples of changes in food systems as a result of the impact of different types of proteins on each other which are called “co-protein effects”. These can be either synergistic or antagonistic. For example, when speaking about polymerization of proteins, a synergistic (or antagonistic) effect occurs when more (or less) proteins polymerize in mixtures of two proteins than expected based on observations made with separate proteins. Even though interactions and reactions between proteins impact on food systems, the occurrence of co-protein effects is not well understood. This is unfortunate as more fundamental knowledge on the impact of egg, soy or whey proteins on protein network formation in wheat dough and later processing steps, can open perspectives for creating cost-effective food products with enhanced properties.

Against the above background, this doctoral work aimed to study interactions and reactions between different types of proteins and their importance for wheat-based food products.

In a first part, the impacts of different co-solvents in extraction and elution media on non-size effects in size-exclusion chromatography were studied. Most techniques for studying proteins require them to be soluble. Egg, soy and whey proteins are soluble in water or salt solutions while wheat gluten proteins are not. Co-solvents are necessary to solubilize gluten proteins but these in some cases interact with size exclusion resins altering the separation and apparent molecular weight distribution. An in-depth study indicated that the use of sodium dodecyl sulfate (SDS) containing medium as extraction and elution medium minimized non-size effects. A method for studying heat-induced covalent network formation of different protein types was developed. It is based on their loss in extractability in SDS containing medium and changes in molecular weight distributions during heating.

In a second part, heat-induced polymerization (100 °C) was studied in model systems for isolated wheat, egg, soy and whey protein (fractions) in water or aqueous ethanol. Proteins polymerized to a larger extent in water than in aqueous ethanol. The results of isolated protein (fractions) were compared with those of their mixtures with gluten proteins. A synergistic co-protein effect was observed in some cases, namely when proteins polymerized to a larger extent in their mixture with gluten than what would be expected based on the weight-averaged results of the isolated proteins. Phase-separation of proteins did not limit the occurrence of synergistic co-protein effects. Both in water and aqueous ethanol different protein types impacted each other's denaturation and/or polymerization. A model was developed to predict co-protein effects between globular and wheat gluten proteins during heating at 100 °C in water. The amount of hydrophobic protein sites and accessible sulfhydryl groups of unfolded globular proteins are key parameters determining co-protein effects in their mixtures with gluten.

In a third part, non-covalent interactions were found to dominate the properties of fresh noodles while covalent cross-links and hydrogen bonds mainly determined the properties of cooked noodles. Ionic and hydrophobic interactions had some impact on cooked noodles but probably by hindering covalent network formation. The addition of whole egg positively impacted the properties of wheat-based noodles even more than that of egg white and egg yolk. Protein (fractions) with a high amount of accessible sulfhydryl groups rapidly initiated disulfide bond formation which reduced the flexibility of the protein network to cope with starch swelling during cooking. However, insufficient cross-linking during cooking lead to noodles with a weak structure and high levels of material leaching into the cooking water.

In conclusion, different types of protein can impact each other's network formation through non-covalent interactions and covalent cross-links. High amounts of accessible sulfhydryl groups and

hydrophobic protein sites in globular protein enhance the rate of gluten protein incorporation in the protein network. However, fast and excessive polymerization can reduce noodle quality. An optimal extent of protein network formation is necessary in wheat-based noodles to obtain superior quality. Addition of whole egg, soy glycinin or bovine serum albumin enhanced the properties of cooked noodles.

# Table of contents

# Symbols and abbreviations

## Symbols

| | |
|---|---|
| $\Theta$ | ellipticity |
| $[y]_0$ | protein extractability in SDS medium at time zero |
| $[y]_{minimal}$ | extractability of proteins which resists polymerization |
| $[y]_t$ | protein extractability in SDS medium at time t |
| k | first-order reaction rate constant of loss in extractability in SDS medium ($min^{-1}$) |
| $T_2$ | transverse relaxation times |
| y | area or SDS-EP in SE-HPLC chromatograms |

## Abbreviations

| | |
|---|---|
| $^1$H NMR | low resolution proton nuclear magnetic resonance |
| ACN medium | acetonitrile/water (1:1, v/v) containing 0.05% (v/v) trifluoroacetic acid |
| ANS | 1-anilino-8-naphthalene sulfonate |
| AU | arbitrary units |
| BSA | bovine serum albumin |

| | |
|---|---|
| CD | circular dichroism |
| CPMG | Carr-Purcell-Meiboom-Gill |
| cv | cultivar |
| DHA | dehydroalanine |
| dm | dry matter |
| DMSO | dimethyl sulfoxide |
| DMSO medium | DMSO:propanol:water (ratio 2:1:1, v/v) |
| DSC | differential scanning calorimetry |
| DTNB | 5,5'-dithiobis(2-nitrobenzoic acid) |
| DTT | dithiothreitol |
| EtPT | aqueous ethanol pretreated |
| FID | free induction decay |
| GS | glutenin subunits |
| HDL | high density lipoproteins |
| HMW-GS | high molecular weight glutenin subunits |
| HPLC | high performance liquid chromatography |
| LAL | lysinoalanine |
| LAN | lanthionine |
| LDL | low density lipoproteins |
| LMW-GS | low molecular weight glutenin subunits |
| MW | molecular weight |
| pI | isoelectric point |
| salt medium | sodium phosphate buffer (0.050 M; pH 7.6) containing 0.4 M sodium chloride |
| SDS | sodium dodecyl sulfate |

| | |
|---|---|
| SDS medium | sodium phosphate buffer (0.050 M; pH 6.8) containing 2.0% (w/v) SDS |
| SDS/DTT medium | SDS medium containing 1.0% (w/v) DTT |
| SDS/urea medium | SDS medium containing 2.0 M urea |
| SDS/urea/DTT medium | SDS medium containing 2.0 M urea and 1.0% (w/v) DTT |
| SDS-EP | proteins extractable in SDS medium |
| SE | size exclusion |
| SH | sulfhydryl |
| SS | disulfide |
| UV | ultraviolet |

# Context and aims

The quality of many food products depends on the type and extent of protein network formation during processing. In some cereal-based food products including cake, cookie, pancake, waffle, bread, tortilla, pasta and noodle systems, wheat proteins coexist with proteins from other sources, *e.g*. eggs and milk. Eggs are relatively expensive with prices fluctuating between 110 and 160 euro/100 kg in Europe (European Commission 2016b). Furthermore, the poultry sector's growth and trends towards intensification and concentration have given rise to a number of environmental concerns (Gerber *et al.* 2007). While soy and whey proteins today are mainly used in feed systems, they can be a cost-effective [recently whey protein isolate costs around 61 euro/100 kg (European Commission 2016a)], sustainable and nutritionally balanced alternative for eggs in the food industry. All these different protein types can impact protein network formation in and quality of wheat-based food products. Synergistic and antagonistic effects between different protein types can change the behavior of food products and their quality. However, the impact of egg, soy or whey proteins on heat-induced network formation and on the quality of wheat-based food products is not well understood.

This work aims to study interactions and reactions between different types of proteins and their impact on wheat-based food products. Both non-covalent interactions (hydrogen bonds, ionic and hydrophobic interactions) and covalent cross-links (mainly disulfide bonds) impact heat-induced protein network formation. The first part of this dissertation, schematically visualized in Figure C.1, is a **literature overview**.

- **Chapter 1** describes the structure and characteristics of the main protein (fractions) in wheat, egg, soy and whey.

- **Chapter 2** summarizes the different types of interactions and reactions between proteins and their importance during heat-induced network formation of those from wheat, egg, soy and whey. Furthermore, it discusses protein network formation in model systems consisting of protein mixtures and it illustrates the impact of different proteins on the quality of wheat-based noodles.

In the **experimental part**, **first**, a size exclusion chromatography method is optimized to study heat-induced covalent network formation for different protein types with various solubility in extraction and elution media.

- In **Chapter 3,** the impact of co-solvents in extraction and elution media on non-size effects in size-exclusion high performance liquid chromatography is investigated. A method for studying differences in heat-induced polymerization is optimized. It is based on (I) losses in protein extractability in a medium in which all unheated proteins are soluble, and (II) changes in molecular weight distributions.

**Second**, protein network formation in mixtures of egg, soy or whey proteins with wheat gluten is evaluated in model systems.

- **Chapter 4** is a study of the denaturation and polymerization of isolated proteins and their mixtures in both water and aqueous ethanol. As such, the impact of thermodynamic (in)compatibility on heat-induced polymerization of mixtures of globular proteins with wheat gliadin, the monomeric fraction of gluten, is investigated. In addition, the impact of ethanol pretreatment on polymerization of isolated proteins and mixtures with gluten is discussed.
- In **Chapter 5**, characteristics of globular proteins are related to their heat-induced polymerization with gluten in water. To that end, heat-induced polymerization of isolated proteins, protein fractions originating from the same source and mixtures of these protein (fractions) with wheat gluten is studied. The key protein characteristics which determine covalent network formation of these proteins with gluten are identified.

**Third**, the impact of egg, soy and whey proteins on protein network formation in and properties of wheat-based noodles is investigated. Noodles are made with from wheat flour, eggs and salt and with a simple production process consisting of mixing, sheeting and slitting.

- **Chapter 6** reports on the investigation of the role of wheat and egg constituents on protein network formation and properties of egg noodles. The impact of covalent cross-links and non-covalent interactions is investigated for fresh and cooked noodles.

- **Chapter 7** deals with the impact of isolated egg, soy and whey proteins on protein network formation in and properties of wheat-based noodles. The impact of non-covalent interactions on fresh and overcooked noodles is outlined using additives which hinder hydrogen bonds, ionic and hydrophobic interactions respectively. Various protein characteristics are linked to the behavior of these proteins during noodle preparation and processing.

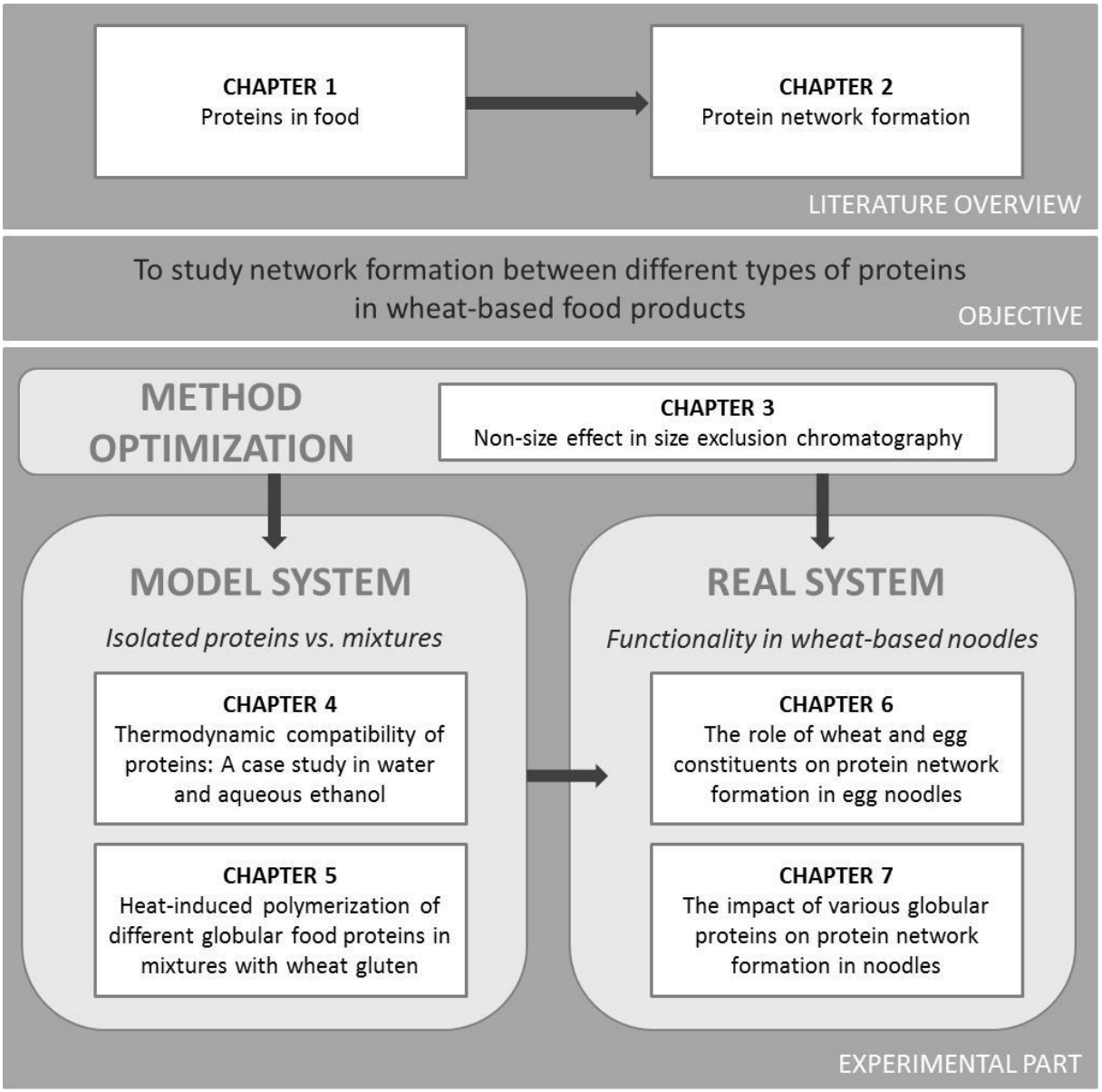



**Figure C.1: Schematic overview and visualization of the relationships between the different chapters in this work**

# PART ONE

# Literature overview

## Chapter 1

### Proteins in food

## Chapter 2

### Protein network formation

# Chapter 1
# Food protein characteristics

## 1.1 Introduction

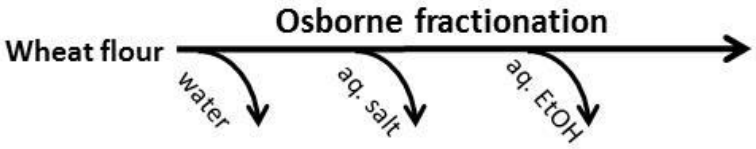
Proteins are not only of nutritional but also of great technological and sensorial importance for food products. For instance, egg, soy, surimi, meat and milk proteins denature and form gels upon heating (Belitz *et al.* 2009). The texture and structure of wheat-based food products such as pasta or bread are to a large degree determined by network formation of the wheat storage proteins (Delcour *et al.* 2012). The amino acid sequences of such proteins differ substantially from those of the globular proteins of *inter alia* eggs, soy and milk. This has important implications for their three dimensional structure and often for their network formation during food processing. In cake, cookie, pancake, waffle, pastry, egg pasta and egg noodle systems wheat proteins coexist with egg and/or milk proteins. Replacing eggs by *e.g.* soy or whey proteins in these food products often reduces consumer acceptance (Lee *et al.* 1993; Ribotta *et al.* 2005; Khouryieh *et al.* 2006; Ratnayake *et al.* 2012). Increasing the knowledge base about the functionality of eggs and other protein sources in food products and their interplay with wheat constituents can yield perspectives for ingredient replacement and the development of new food systems. However, insight in the structure-function relationship of proteins in complex systems remains limited. In this context, it is useful to discuss the most striking structural characteristics of wheat, egg, soy and whey proteins. This is what is done in the first chapter of this literature survey.

## 1.2 Wheat proteins

Commercial wheat flour consists of starch (*ca.* 70-75%), water (*ca.* 14%), proteins (*ca.* 10-12%), non-starch polysaccharides (*ca.* 2-3%) and lipids (*ca.* 2%) (Goesaert *et al.* 2005). Common wheat (*Triticum aestivum* L.) flour can originate from hard or soft wheat cultivars (cvs). Hard wheat flour has superior protein content and bread making quality when compared to soft wheat flour. Wheat flour protein quality in the context of bread making is determined by the molecular structure of its major proteins and the interactions between them (Bushuk 1998). Osborne (1907) distinguished between albumin, globulin, gliadin and glutenin fractions by sequentially extracting wheat flour with water, dilute salt solution, aqueous ethanol and acid/alkaline solutions, respectively. The residue remaining after the aqueous ethanol step is called glutenin (Belitz *et al.* 2009). Proteins belonging to the same Osborne fraction can differ in structure and functionality. The Osborne fractionation is still commonly used because it is relatively simple, reproducible and relevant in the context of food protein functionality (Goesaert *et al.* 2005). About 15-20% of the total wheat proteins are classified as albumins and globulins while the remainder is gliadin and glutenin. Upon mixing with water, gliadin and glutenin form gluten, which gives wheat dough unique viscoelastic properties (Veraverbeke and Delcour 2002).

### 1.2.1 Wheat albumin and globulin

The majority of albumins and globulins are monomeric and have a molecular weight (MW) lower than 25 k while some have MWs between 60 k and 70 k (Singh *et al.* 1990; Veraverbeke and Delcour 2002). Furthermore, β-amylases (albumins) and triticins (globulins) can form larger polymers stabilized by intermolecular disulfide (SS) bonds. Triticins are located in the protein bodies in the starchy endosperm (Gianibelli *et al.* 2001). This in contrast to other albumins and globulins which are mostly located in the outer layers of the wheat kernel (Wrigley and Bietz 1988). Albumins and globulins are usually structural or metabolic (enzymes and enzyme inhibitors) proteins (Veraverbeke and Delcour 2002). These non-gluten proteins have better nutritional values than gluten proteins as they contain higher levels of lysine, tryptophan and methionine (Figure 1.1) (Delcour and Hoseney 2010).




| | Albumin | Globulin | Gliadin | Glutenin |
|---|---|---|---|---|
| Asparagine/ aspartic acid | 4.2 | 9.7 | 7.7 | 2.7 | 3.7 |
| Threonine | 3.2 | 3.8 | 4.6 | 2.3 | 3.6 |
| Serine | 6.6 | 6.2 | 6.6 | 5.9 | 7.3 |
| Glutamine/ glutamic acid | 31.1 | 20.9 | 15.2 | 37.1 | 30.1 |
| Proline | 12.6 | 9.3 | 6.9 | 16.6 | 11.9 |
| Glycine | 6.1 | 6.9 | 8.3 | 2.9 | 7.9 |
| Alanine | 4.3 | 6.9 | 7.5 | 2.8 | 4.4 |
| Cysteine | 1.8 | 3.2 | 3.6 | 2.2 | 1.4 |
| Valine | 4.9 | 6.0 | 6.8 | 4.2 | 4.8 |
| Methionine | 1.4 | 1.6 | 2.0 | 1.1 | 1.3 |
| Isoleucine | 3.8 | 3.3 | 3.8 | 4.1 | 3.5 |
| Leucine | 6.8 | 6.4 | 7.3 | 6.9 | 6.9 |
| Tyrosine | 2.3 | 2.8 | 2.9 | 2.0 | 2.4 |
| Phenylalanine | 3.8 | 3.1 | 3.1 | 4.6 | 3.6 |
| Histidine | 1.8 | 1.8 | 2.4 | 1.7 | 1.8 |
| Lysine | 1.8 | 3.0 | 4.0 | 0.8 | 2.1 |
| Arginine | 2.8 | 4.0 | 6.4 | 1.7 | 2.7 |
| Tryptophan | 0.7 | 1.1 | 0.9 | 0.4 | 0.6 |

**Figure 1.1: Osborne fractionation of wheat flour with water, aqueous salt solution (aq. salt) and aqueous ethanol (aq. EtOH). The average amino acid compositions of wheat flour, albumin, globulin, gliadin and glutenin fractions are expressed in mole % (Belitz *et al.* 2009). Essential amino acids are colored in grey.**

## 1.2.2 Wheat gliadin

Gluten proteins contain high levels of glutamine and the nonpolar amino acids proline and glycine but low levels of ionic amino acids (Gianibelli *et al.* 2001; Delcour and Hoseney 2010). Gluten consists of roughly equal proportions of monomeric gliadin and polymeric glutenin (Wieser 2007). Gliadins have MWs between *ca.* 30 k and *ca.* 75 k (Gianibelli *et al.* 2001) and can be subdivided based on their mobility in gel electrophoresis and their amino acid compositions in α-, γ-, ω5- and ω1,2-gliadins (Wieser 2007). α-Gliadins and γ-gliadins have lower MWs (*ca.* 28 k-35 k) than ω5-gliadins (*ca.* 50 k) and ω1,2-gliadins (*ca.* 40 k). The primary structure of ω-gliadin consists almost entirely of repetitive sequences rich in glutamine and proline with small non-repetitive N- and C-terminal domains (Figure 1.2). A total of about 80% of the amino acids in ω-gliadin are glutamine, proline and phenylalanine. The structures of α- and γ-gliadins contain less of these amino acids which similarly form repetitive sequences in their N-terminal domain rich in β-turns. The sequences in the C-terminal

domain of α- and γ-gliadins are non-repetitive, rich in α-helices, and contain cysteine residues. Usually, α-gliadins have six while γ-gliadins have eight cysteine residues which form respectively three and four intramolecular SS bonds. ω-Gliadin lacks cysteine residues. Apparently, due to point mutations, some gliadins contain an odd number of cysteine residues (Wieser 2007; Delcour *et al.* 2012). It is assumed that the tertiary structure of ω-gliadin is a stiff coil while that of α-gliadin is compact and less regularly structured. γ-Gliadin is regarded to be an extended-spiral (Delcour *et al.* 2012). During heating, the structure of ω-gliadins is mainly stabilized by hydrophobic interactions while those of α- and γ-gliadins are mainly stabilized by SS and hydrogen bonds (Tatham and Shewry 1985). Of particular importance is that wheat gluten proteins do not show endothermic peaks during heating when analyzed by differential scanning calorimetry (DSC) (Erdogdu *et al.* 1995).

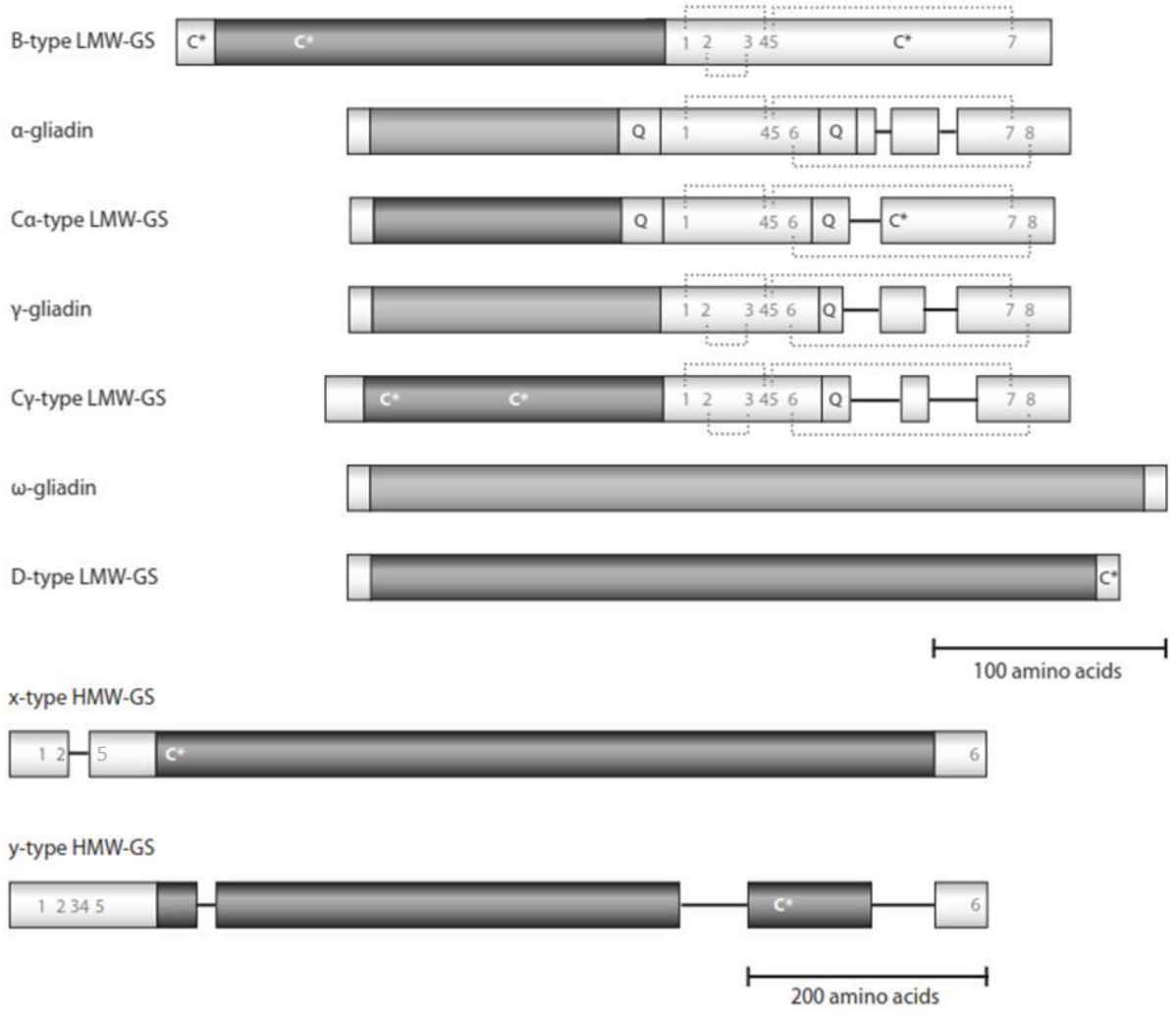



**Figure 1.2: Schematic representation of different types of gluten proteins. As the structure and the position of cysteine residues of some C-LMW-GS highly corresponds with α- and γ-gliadins, they are called Cα- and Cγ-LMW-GS. Repetitive domains are grey and non-repetitive domains are white. Positions of conserved cysteine residues are indicated with Arabic numbers and those of unconserved cysteine residues with C*. Dotted lines indicate intramolecular disulfide (SS) bonds between cysteine residues. Q represents a polyglutamine sequence. Homologue polypeptide regions are aligned based on their amino acid sequence (Delcour *et al.* 2012).**

### 1.2.3 Wheat glutenin

The MW of glutenin ranges from 500 k to more than 10 million. Glutenin is composed of subunits (GS) interconnected by SS bonds. After reducing the SS bonds, the released GS are as soluble in aqueous ethanol as gliadin (Wieser 2007). GS can be subdivided in four groups based on decreasing mobility in gel electrophoresis. Group A contains high MW GS (HMW-GS) while groups B, C and D contain low MW GS (LMW-GS). About 60% of glutenin are LMW-GS (Gianibelli *et al.* 2001). B- (MWs 42 k-51 k) and C-LMW-GS (MWs 30 k-40 k) are homologous to α- and γ-gliadins while the structure of D-LMW-GS (MWs 55 k-70 k) is related to that of ω-gliadins (Figure 1.2) (Lindsay and Skerritt 1999). However, GS contain not only intramolecular SS bonds but also free cysteine residues which can form intermolecular SS bonds. D-LMW-GS have one free cysteine residue. B-LMW-GS contain six highly conserved and one or two variable cysteine residues (Figure 1.2) (Shewry and Tatham 1997). C-LMW-GS highly resemble α- or γ-gliadins and are called Cα- and Cγ-LMW-GS. The former contain six and the latter eight conserved cysteine residues (Lew *et al.* 1992). Additional cysteine residues can be found as indicated in Figure 1.2 (Okita *et al.* 1985; Köhler *et al.* 1993; D'Ovidio *et al.* 1995). While the HMW-GS are less abundant, they have been well-studied as their composition in a given wheat cv is related to its bread making quality (Payne 1987). Each wheat cv contains three to five HMW-GS which can be grouped in x- (MWs 83 k-88 k) and y-type (MWs 67 k-74 k). HMW-GS are referred to by their genome, x-or y-type and the order of mobility in gel electrophoresis according to Payne *et al.* (1981). They have a central repetitive domain rich in glutamine, proline and glycine which mainly forms β-strands. It is flanked by N- and C-terminal domains which contain charged and cysteine residues and mainly form α-helices. Generally, y-type HMW-GS contain higher levels of cysteine than x-type HMW-GS. HMW-GS 1Dx5 is the only x-type HMW-GS in which a non-conserved cysteine residue has been found. This in contrast to y-type HMW-GS, which contain such cysteine residues (Veraverbeke and Delcour 2002; Wieser 2007).

## 1.3 Hen egg proteins

Hen (*Gallus gallus domesticus*) eggs are used such as well as in a wide range of food products. They have high nutritional (Table 1.1) and techno-functional value (Mine 2002). Eggs are a good source of proteins, vitamins and minerals. The earlier negative connotation with consumers resulting from the presence of cholesterol has partially been rectified by studies indicating that eggs do not have negative effects on serum cholesterol levels (Hu *et al.* 2001). Whole egg consists of two edible fractions, *i.e.* egg white and egg yolk, which are present in a ratio of 2 to 1 (Powrie and Nakai 1985). Egg white and egg yolk have unique foaming and emulsifying properties, respectively. Both egg fractions form gels upon heating (Mine 2002). About 92% of the egg white solids (*ca.* 12%) consist of

protein. In contrast, egg yolk solids (*ca.* 53%) contain *ca.* 62.5% lipids and *ca.* 33% protein (Powrie and Nakai 1985). Table 1.2 shows the compositions of whole egg and its fractions.

**Table 1.1: Amino acid compositions of whole egg, egg white, egg yolk, soy glycinin, soy β-conglycinin and whey expressed in mole % (Belitz *et al.* 2009). Essential amino acids are colored in grey.**

| | Whole egg | Egg white | Egg yolk | Glycinin | β-Conglycinin | Whey |
|---|---|---|---|---|---|---|
| Asparagine/ aspartic acid | 5.3 | 5.7 | 5.0 | 13.3 | 11.7 | 9.5 |
| Threonine | 7.0 | 6.2 | 7.7 | 3.9 | 3.6 | 8.2 |
| Serine | 11.5 | 9.5 | 10.5 | 5.4 | 6.4 | 6.0 |
| Glutamine/ glutamic acid | 2.0 | 2.1 | 1.5 | 16.6 | 16.6 | 12.2 |
| Proline | 13.8 | 15.5 | 13.7 | 6.4 | 6.3 | 4.4 |
| Glycine | 5.9 | 6.2 | 6.1 | 7.2 | 6.3 | 5.4 |
| Alanine | 3.5 | 3.1 | 3.4 | 5.5 | 5.8 | 7.1 |
| Cysteine | 7.0 | 6.8 | 6.7 | 1.6 | 1.7 | 2.9 |
| Valine | 9.6 | 9.5 | 9.5 | 5.9 | 5.6 | 7.4 |
| Methionine | 4.6 | 5.1 | 5.8 | 1.6 | 0.4 | 1.9 |
| Isoleucine | 3.1 | 3.7 | 2.6 | 4.9 | 5.3 | 6.2 |
| Leucine | 5.6 | 6.1 | 4.5 | 7.4 | 8.7 | 10.4 |
| Tyrosine | 3.0 | 2.6 | 3.2 | 3.1 | 2.7 | 2.7 |
| Phenylalanine | 5.5 | 5.3 | 6.4 | 4.7 | 4.7 | 2.9 |
| Histidine | 3.3 | 3.6 | 4.3 | 1.9 | 2.1 | 1.8 |
| Lysine | 1.4 | 1.3 | 1.3 | 4.5 | 6.0 | 7.6 |
| Arginine | 3.2 | 3.0 | 3.5 | 6.1 | 5.9 | 2.2 |
| Tryptophan | 4.7 | 4.8 | 4.4 | - | - | 1.2 |

**Table 1.2: Composition of whole egg, egg white and egg yolk expressed on wet basis (Powrie and Nakai 1985).**

| Constituent | Whole egg | Egg white | Egg yolk |
|---|---|---|---|
| Solids (%) | 25-26.5 | 11.1 | 52.3-53.5 |
| Proteins (%) | 12.8-13.4 | 9.7-10.6 | 15.7-16.6 |
| Lipids (%) | 10.5-11.8 | 0.03 | 31.8-35.5 |
| Carbohydrates (%) | 0.3-1.0 | 0.4-0.9 | 0.2-1.0 |
| Ash (%) | 0.8-1.0 | 0.5-0.6 | 1.1 |

### 1.3.1 Egg white

Egg white contains more than 40 different proteins of which ovalbumin (*ca.* 54%) dominates its gelling properties. Carbohydrates in egg white (Table 1.2) exist either in free form or bound to proteins (Mine 2002). Ovalbumin is the only egg white protein which has free sulfhydryl (SH) groups (Powrie and Nakai 1985). These can form intermolecular SS bonds and thereby stabilize gels (Mine 1995). Table 1.3 gives an overview of the most important proteins in egg white with some physical

and chemical characteristics. The isoelectric point (pI) of ovalbumin (Table 1.3) is *ca.* 4.5. At about such pH, egg white protein has lower solubility than at higher pH values (Machado *et al.* 2007).

**Table 1.3: The most abundant proteins in egg white with their molecular weight (MW), isoelectric point (pI), amount of sulfhydryl (SH) groups and disulfide (SS) bonds per molecule. Also listed are their denaturation temperatures ($T_d$). All values were from Powrie and Nakai (1985) except where indicated.**

| Egg white protein | Abundance of egg white protein (% dm) | MW (k) | pI | SH groups | SS bonds | $T_d^*$ (°C) |
|---|---|---|---|---|---|---|
| Ovalbumin | 54.0 | 44.5 | 4.5 | 4 | 1 | 84.0 |
| Ovotransferrin | 12.0 | 76.0 | 6.1 | 0 | 15 | 61.0 |
| Ovomucoid | 11.0 | 28.0 | 4.1 | 0 | 8 | 79.0 |
| Ovoglobulin | *ca.* 8 | 49.0 | 4.8-5.1 | 0 | ≥1□ | 92.5 |
| Ovomucin | 3.5 | 5.5-8.3 x$10^3$ | 4.5-5.0 | 0 | ≥1Δ | - |
| Lysozyme | 3.4 | 14.3 | 10.7 | 0 | 4 | 75.0 |

*Determined at pH 7.0 on isolated proteins (Donovan *et al.* 1975)
Δ (Alleoni 2006)
□ (Li-Chan *et al.* 1995)
- not determined

### 1.3.1.1 Ovalbumin

Ovalbumin is a phosphoglycoprotein containing 385 amino acids and *ca.* 3.5% carbohydrates (Nisbet *et al.* 1981; Alleoni 2006). Half of its amino acids are hydrophobic and one third is ionic (Huopalathi *et al.* 2007). Based on the degree of phosphorylation of two serine residues (Ser-68 and Ser-344), three groups can be distinguished by gel electrophoresis. The ratio of serine residues with two, one and no phosphate groups per molecule of ovalbumin is 85:12:3. Ovalbumin contains six cysteine residues of which two are involved in an SS bond (Powrie and Nakai 1985). Three cysteine residues are weakly reactive in native ovalbumin, and one is only reactive after denaturation.

During egg storage, ovalbumin is converted into its more thermostable form **S-ovalbumin**. Cool storage increases the level of S-ovalbumin from 5% in fresh eggs to 81% in egg stored for six months (Alleoni 2006). Alkaline pH and higher storage temperatures increase the conversion of ovalbumin into S-ovalbumin which follows first-order kinetics (Donovan and Mapes 1976) and involves isomerization of three serine residues (Ser-164, Ser-236 and Ser-320) into their D-amino acids (Yamasaki *et al.* 2003). It increases the denaturation temperature of ovalbumin with *ca.* 8 °C (Donovan and Mapes 1976). However, only isomerization of serine residues 164 and 320 contribute to the thermostability of S-ovalbumin (Takahashi *et al.* 2010). No other differences in amino acid compositions between ovalbumin and S-ovalbumin have been demonstrated (Mine 1995).

#### 1.3.1.2 Ovotransferrin

Ovotransferrin, also called conalbumin, is a glycoprotein of 686 amino acids. It contains neither phosphorous nor free SH groups (Powrie and Nakai 1985). It consists of two lobs which can each bind a metal ion (Alleoni 2006; Huopalathi *et al.* 2007). Ovotransferrin can bind different levels (two, one or no ions per molecule) of $Fe^{3+}$, $Al^{3+}$, $Cu^{2+}$ or $Zn^{2+}$ along with two $CO_3^{2-}$ or $HCO_3^-$ ions. Binding of two ions per molecule forms a heat-stable protein-ion complex (Powrie and Nakai 1985; Huopalathi *et al.* 2007). The binding of $Al^{3+}$ increases its denaturation temperature at pH 7.0 from 61.0 °C to 73.5 °C (Donovan *et al.* 1975). Furthermore, protein-metal ion complexation increases the resistance of ovotransferrin to denaturation by pressure, enzymes and denaturing agents (Alleoni 2006). Ovotransferrin has been reported to inhibit bacteria (Mine 1995).

#### 1.3.1.3 Ovomucoid

Ovomucoid is a glycoprotein which contains *ca.* 20-25% carbohydrates. It consists of three separate domains each cross-linked by three SS bonds (Powrie and Nakai 1985). It inhibits trypsin activity (Mine 1995) and can be heated at 100 °C under acidic conditions without any notable changes in properties. However, it is sensitive for denaturation under alkaline conditions (Deutsch and Morton 1961).

#### 1.3.1.4 Ovoglobulin

Little is known about ovoglobulins in egg white. Two groups (G2 and G3) with similar amino acid and carbohydrate compositions can be distinguished. Ovoglobulins are considered to have good foaming properties but their roles have not been clarified (Mine 1995).

#### 1.3.1.5 Ovomucin

Ovomucoid is a glycosulfiprotein. It contains two subunits: α and β-ovomucin (Huopalathi *et al.* 2007). Less carbohydrate is connected to α-ovomucin (*ca.* 15%) than to β-ovomucin (*ca.* 50%) (Powrie and Nakai 1985). The polypeptide has 2,087 amino acids. It is the largest egg white protein (Table 1.2) (Huopalathi *et al.* 2007). It contains sulfate esters and high levels of cysteine residues which are all involved in intramolecular SS bonds (Alleoni 2006). Ovomucin is important for the structure of egg white as it is highly viscous and has a gel-like nature at room temperature (Mine 1995). Purified ovomucin is resistant to heat-induced denaturation (Powrie and Nakai 1985). It is insoluble at neutral pH in absence of denaturing agents but can be solubilized by mechanical treatments in mildly alkaline buffers (Kato *et al.* 1985).

#### 1.3.1.6 Lysozyme

Lysozyme, also known as muramidase or initially as ovoglobulin G1, in some territories can be used as food preservative as it hydrolyses β-1-4 glycosidic bonds of Gram-negative bacteria. It is the smallest egg white protein (Table 1.1). Its pI is higher than that of other egg white proteins. It consists of two domains connected through an α-helix and contains 129 amino acids of which eight cysteine residues are involved in SS bonds. The four intramolecular SS bonds in this small protein make its tertiary structure highly compact and stable. All of the polar groups are located outside the protein while the majority of hydrophobic groups are gathered in the protein core (Huopalathi *et al.* 2007). After moderate heat or pH treatments, lysozyme can refold and regain its enzymatic activity. Above a critical concentration or at high temperatures and heating times, lysozyme aggregates which makes refolding impossible (Wu *et al.* 2015).

### 1.3.2 Egg yolk

Egg yolk is a complex emulsion (Mine 2002). It consists of five major constituents [expressed on egg yolk dry matter (dm)]: low density lipoproteins (LDL, ca. 68%), high density lipoproteins (HDL, *ca.* 16%), livetins (*ca.* 10%), phosvitin (*ca.* 4%) and some minor proteins (*ca*. 2%). About 58% of egg yolk proteins appear as lipoproteins. Lipids are exclusively present in lipoprotein assemblies (Huopalathi *et al.* 2007). The egg yolk lipid fraction consists of triacylglycerol (*ca.* 66%), phospholipid (*ca.* 28%) and cholesterol (*ca.* 5%) (Powrie and Nakai 1985). Carotenoids (< 1% of yolk lipids) are responsible for the yellow color (Huopalathi *et al.* 2007).

Egg yolk consists of plasma which contains mainly LDL, livetins and minor proteins and granules that are made up by HDL, phosvitin and only *ca.* 2% LDL (Powrie and Nakai 1985). The LDL in egg yolk plasma (17-60 nm) and the egg yolk granules (0.3-2 µm) appears in the form of natural nano- and micro-assemblies, respectively (Figure 1.3). LDL contains large quantities of lipids (*ca.* 61% of egg yolk lipids) whereas granules are mostly stacked with proteins (*ca.* 47% egg yolk proteins and *ca.* 7% egg yolk lipids) (Powrie and Nakai 1985; Anton 2013). HDL and phosvitin are linked by phosphocalcic bridges which make the granule compact, poorly hydrated and resistant for enzymes and heat. Replacing $Ca^{2+}$ by $Na^{+}$ at an ionic strength of 0.3 M disrupts the phosphocalcic bridges and increases the solubility of egg yolk granules in water to 80%. In soluble state (at high ionic strength and neutral pH), phosvitin and HDL assemble in micelles (100-200 nm) (Anton 2013).

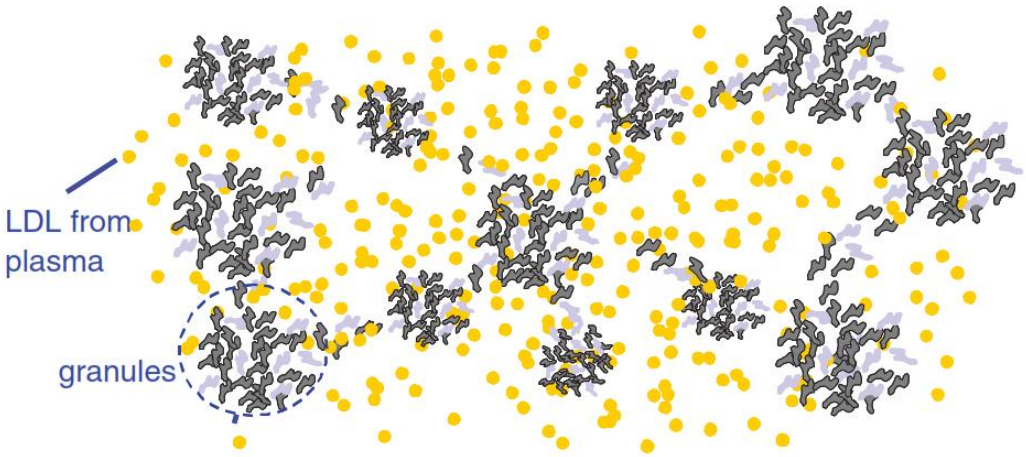



**Figure 1.3: Schematic visualization of egg yolk. Low-density lipoproteins (LDL) and egg yolk granules, respectively nano- and micro-structures, are dispersed in an aqueous solution, the egg yolk plasma (Anton 2013).**

### 1.3.2.1 Low density lipoproteins

LDL, sometimes called lipovitellenin, are soluble in aqueous solutions. As their name implies, they have low density (0.982 kg/m³). LDL has a lipid core in a liquid state which consists of triacylglycerol and cholesterol esters (Figure 1.4). This core is surrounded by a monofilm of amphiphilic phospholipids, apoproteins and cholesterol. The latter increases the rigidity of the structure.

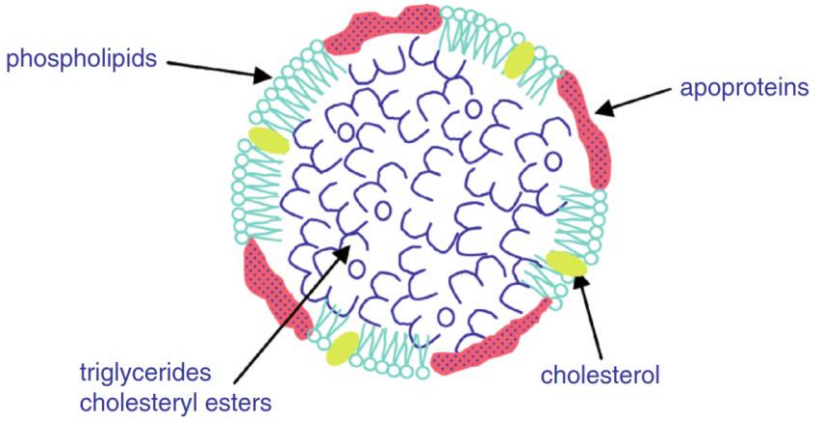



**Figure 1.4: Schematic visualization of a low density lipoprotein (Huopalathi *et al.* 2007).**

Six different apoproteins can be distinguished in LDL of which the MW ranges between 15 k and 130 k. The largest apoprotein is the most abundant (*ca*. 70%) while the smallest one counts for *ca*. 20% of the apoproteins. Their pI range from 6.3 to 7.5. Apoproteins are glycosylated and contain *ca.* 40% hydrophobic amino acids. Apovitellenin I is the only apoprotein of which the structure has been well characterized. Apovitellenin I is a small homodimer with both monomers (MW ca. 9 k) connected through a SS bond (Huopalathi *et al.* 2007). Vitellenin of LDL denatures around 72 °C (Anton *et al.* 2000).

#### 1.3.2.2 Livetins

Three water soluble livetin fractions are distinguished. These are α-, β- and γ-livetins (ratio 2:5:3). The α-livetin fraction has been further identified as chicken serum albumin (MW 70 k, pI 4.3-5.7), β-livetin as α-2-glycoprotein (MW 45 k) and γ-livetin as immunoglobulin Y (MW 167,250) (Huopalathi *et al.* 2007). The denaturation temperature of α-livetin (*ca*. 72 °C) is higher than that of γ-livetin (ca. 62 °C) but lower than that of β-livetin (Le Denmat *et al.* 1999).

#### 1.3.2.3 High density lipoproteins

HDL have higher density than LDL and close to that of proteins. HDL is a dimer (MW ca. 400 k) of which each monomer consist of about five main glycosylated apoproteins with MWs ranging from 35 k to 100 k. Two groups, α- and β- HDL (ratio 1:1.5), can be separated with ion exchange chromatography. The former is more acidic and less water soluble than the latter. Further, they have similar chemical compositions and contain about 30% hydrophobic amino acid residues. Each monomer has a cavity in the form of a funnel which can contain about 35 molecules of phospholipid in a monolayer. This phospholipid monolayer can further enclose triacylglycerol in a cavity. Five SS bonds and numerous ionic and hydrophobic interactions maintain this well-ordered structure (Huopalathi *et al.* 2007). α-HDL denatures around 76 °C while β-HDL seems heat-resistant (Anton *et al.* 2000).

#### 1.3.2.4 Phosvitin

Phosvitin is a phosphoglycoprotein of which 90% of the serine residues (*ca.* 50% of the primary structure) are phosphorylated. This high degree of phosphorylation increases its affinity for metal ions. About 90% of the iron ions in egg yolk are bound by phosvitin. Phosvitin occurs in an α- and a β-form with MWs of 160 k and 190 k respectively. Each phosvitin type is a polypeptide containing subunits with MWs ranging from 35 k to 45 k. The phosphoserine residues are located in the central domain resulting in a hydrophilic zone flanked by small hydrophobic N- and C-terminal domains. In contrast to other egg yolk proteins, phosvitin contains little if any cysteine residues and SS bonds (Huopalathi *et al.* 2007). Similar to β-HDL, α- and β-phosvitin seem heat-resistant (Anton *et al.* 2000).

## 1.4 Soy proteins

Soybeans have high protein contents (*ca*. 40% on dm) and well-balanced amino acid compositions (Table 1.1) (Nishinari *et al.* 2014). Following Osborne fractionation, *ca.* 10% are albumins while *ca.* 90% are globulins. About 80% of the soy proteins are extractable with salt containing medium at pH 6.8 (Belitz *et al.* 2009). The main storage proteins glycinin and β-conglycinin are easily separated by precipitation at pH 4.5 to 4.8 from proteins such as lipoxygenase, β-amylase, lectin and trypsin

inhibitors which are soluble in acid containing medium. The ratio of β-conglycinin to glycinin is cv dependent and varies between 0.5 and 1.3 (Nishinari *et al.* 2014).

### 1.4.1 Glycinin

A glycinin subunit consists of an acidic (pI *ca*. 5) and a basic (pI *ca*. 8.2) polypeptide which are connected through a single SS bond (Staswick *et al.* 1984; Belitz *et al.* 2009). At least five basic (MW *ca*. 35 k) and six acidic (MW *ca*. 20 k) polypeptides have been identified (Staswick *et al.* 1984). Environmental conditions including ionic strength and pH impact the structure and denaturation temperature of glycinin.

At ambient temperatures and pH 7.6, glycinin subunits form a hexameric complex (MW *ca.* 360 k) whereas at pH 3.8 and/or low ionic strength glycinin dissociates in two trimeric complexes (MW *ca.* 180 k) (Lakemond *et al.* 2000b). The trimeric form of glycinin denatures at lower temperature (*ca*. 73 °C) than its hexameric form (*ca*. 80 °C). Hexameric glycinin [pI 4.5 to 6.0 (Liu *et al.* 1999)] has a compact structure with *ca.* 20 SS bonds and no free SH groups (Draper and Catsimpoolas 1978). In this conformation, the monomers associate by non-covalent interactions and form a hollow cylinder (Figure 1.5) (Badley *et al.* 1975). At different ionic strengths, the acidic polypeptides are preferably located at the outside of the glycinin complex while the basic polypeptides, which contain more hydrophobic amino acids, are located in the inner part (Lakemond *et al.* 2000b).

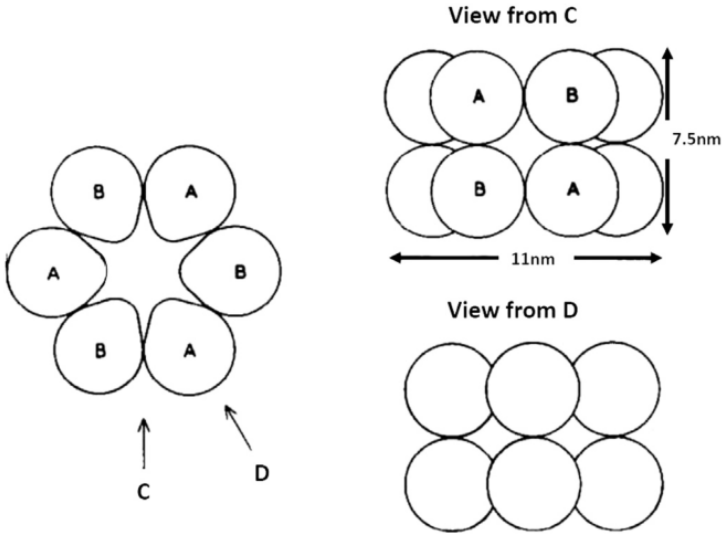



**Figure 1.5: Schematic visualization of glycinin in its hexameric conformation with acidic (A) and basic (B) polypeptides (Badley *et al.* 1975; Nishinari *et al.* 2014).**

### 1.4.2 β-Conglycinin

β-Conglycinin is a glycoprotein with a MW of about 180 k to 210 k. It consists of three major subunits: α (MW *ca.* 68 k, pI 4.9), α' (MW *ca.* 72 k, pI 5.7-6.0) and β (MW *ca.* 52 k, pI 5.2). The latter contains more hydrophobic amino acids than the α and α' subunits. The β subunits lack cysteine residues while the α and α' subunits both contain one. The subunits can associate mainly by hydrogen and hydrophobic interactions and thereby form six different trimers: $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha_2\alpha'$ and $\alpha_3$ (Singh *et al.* 2015).

## 1.5 Bovine whey proteins

Cow (*Bos taurus*) milk contains about 3.2% milk proteins of which 2.6% is casein and 0.6% is whey protein (Belitz *et al.* 2009). During cheese production, casein aggregates while whey remains soluble. Whey is concentrated to protein contents exceeding 90% for different food applications (*e.g.* vegetarian products and nutritional drinks). Its proteins have high nutritional value (Table 1.1) and good gelling and emulsifying properties (Foegeding *et al.* 2002). The major whey proteins are β-lactoglobulin (*ca.* 50%), α-lactalbumin (*ca.* 20%) and bovine serum albumin (BSA, *ca*. 10%) (Boye *et al.* 1996; de la Fuente *et al.* 2002).

### 1.5.1 β-Lactoglobulin

β-Lactoglobulin is a small protein (MW 18 k) with five cysteine residues of which four form intramolecular SS bonds. The only free SH group is buried in the protein structure. It becomes available for reaction after denaturation. The monomer is stable below pH 3.5 and above pH 7.5. Between these pH values, β-lactoglobulin aggregates reversibly into oligomers (Belitz *et al.* 2009). At pH values above its pI 5.4, β-lactoglobulin usually appears as a dimer (de la Fuente *et al.* 2002). Between pH 3.5 and 5.5, four dimers aggregate into an octamer. Above pH 9.0 or during heating irreversible denaturation occurs (Sawyer and Kontopidis 2000). β-Lactoglobulin denatures around 72 °C (Boye and Alli 2000).

### 1.5.2 α-Lactalbumin

α-Lactalbumin is a nearly spherical compact protein with a MW of 14 k and a pI about 4.3. It contains four SS bonds and no free SH groups (de la Fuente *et al.* 2002; Belitz *et al.* 2009). The amino acid composition and sequence resemble those of lysozyme (Section 1.3.1.6). Although α-lactalbumin denatures at *ca.* 65 °C it appears to be heat resistant as it easily refolds. The binding of a $Ca^{2+}$ ion increases its thermal stability and is required for refolding after denaturation (Brew 2013). In absence of $Ca^{2+}$ ions, α-lactalbumin denatures around 35 °C (Boye and Alli 2000).

### 1.5.3 Bovine serum albumin

Originally, BSA was a blood plasma protein transporting ligands through the circulatory system. Throughout evolution about 70% to 80% of the amino acid composition of BSA has changed due to the development of specific binding sites. However, the tertiary structure and the SS bond pattern remained highly conserved (Bujacz 2012). BSA is a globular 'heart-shaped' protein with a MW of 66 k and contains 583 amino acids (Hirayama *et al.* 1990). This single polypeptide is composed of three similar domains each formed by six helices (Hirayama *et al.* 1990; Borzova *et al.* 2016). It has 17 intramolecular SS bonds and one free SH groups buried in a hydrophobic pocket of domain I (Militello *et al.* 2003). These intramolecular SS bonds provide rigidity to each domain but allow the structure to change under different environmental conditions (*e.g.* pH, ionic strength and heat). Seven isomeric forms with differences in α-helical content exist at different pH values. For example, at pH < 4, BSA reversibly loses α-helical structure and changes into an 'extended' conformation (Curvale *et al.* 2008) of much higher length (26.7 nm) than that of the 'normal' compact state (8.3 nm) at pH 4 to 9 (Jachimska and Pajor 2012). BSA has a pI of 4.7. It is water soluble and contains about 185 ionized groups per molecule at pH 7 (Giancola *et al.* 1997). While both charged and apolar residues are located at the surface, the core is mainly hydrophobic (Bhattacharya *et al.* 2011). The denaturation temperature in water without ligand binding is *ca*. 56 °C (Michnik 2003).

Thus, wheat, egg, soy and whey proteins are all complex mixtures of proteins with different protein structures, characteristics and nutritional values. Their functionality in food products is often related to their ability to interact with other food constituents and more specifically with other proteins. Network formation between similar and different types of proteins is described in Chapter 2.

# Chapter 2
# Protein network formation

## 2.1 Introduction

A key concept in protein science is that conformation and function are inseparable. Protein aggregation is related to neurodegenerative diseases (Chiti and Dobson 2006) and sickle cell anemia (De Llano and Manning 1994), but also to texture and structural properties of food products (Singh 1991; Totosaus *et al.* 2002). Food processing unit operations, *e.g.* those involving exposure to heat, pressure and/or solvents, often induce changes in the conformation of proteins and exposure of reactive groups. Heat-induced denaturation of globular proteins, *i.e.* the transformation of the native to a less ordered conformation, changes protein functionality and often induces gelling. Protein aggregation involves both non-covalent interactions and covalent cross-links. The latter are mainly SS bonds (Mine 1995; Foegeding and Davis 2011). Cross-links are either intramolecular or intermolecular (Gerrard 2002). Polymerization of wheat gluten proteins, mainly through SS bond formation and reshuffling, positively impacts the quality of many cereal-based products (Delcour *et al.* 2012). In *inter alia* cake and egg noodles, wheat gluten and globular proteins co-exist. Co-protein effects due to interactions and reactions between different protein types can impact complex food systems (Erickson *et al.* 2012; Rombouts *et al.* 2012c).

In this Chapter, different types of protein interactions and cross-links and their impact on heat-induced protein network formation for proteins within one protein source are discussed.

Furthermore, the impact of different types of proteins on each other's network formation and functionality is discussed for model systems and wheat-based noodles.

## 2.2 Non-covalent interactions and covalent cross-links

Food processing can involve high temperature treatments, adjustment of pH and/or exposure to oxidizing agents or enzymes. Such conditions can induce cross-linking which changes the structure of proteins and – in some cases – the entire food systems (Gerrard 2002). The covalent cross-links that dominate protein network formation in some food systems are SS bonds, dehydroalanine (DHA)-derived and isopeptide cross-links (Singh 1991). Furthermore, non-covalent interactions (hydrophobic, ionic, van der Waals interactions and hydrogen bonds) need to be considered (Belitz *et al.* 2009).

### 2.2.1 Non-covalent interactions

Often, covalent protein networks in food are superimposed by low energy non-covalent interactions (Wieser 2007). **Ionic interactions** are attractive or repulsive between respectively oppositely or equally charged molecules. Histidine, lysine and arginine are mostly positively charged when in protein chains while glutamic and aspartic acid residues in proteins usually bear a negative charge.

**Hydrogen bonds** occur when a hydrogen atom is shared between a hydrogen donor and a hydrogen acceptor. Partially electronegative atoms such as nitrogen and oxygen pull electron density away from a covalently bound hydrogen atom, giving the latter a partially positive charge, which in its turn attracts another partially electronegative atom. The polar uncharged amino acids serine, threonine, asparagine, tyrosine and glutamine participate in hydrogen bonds. These relatively weak interactions (*ca.* 4-20 kJ/mol) are important in food protein networks with water as common solvent.

**Hydrophobic interactions** refer to the association of nonpolar groups to minimize contact with polar groups and solvents. Amino acids other than the charged and polar ones can be involved in hydrophobic interactions.

**Van der Waals interactions** are weak attractions (*ca.* 2-4 kJ/mol) between atoms due to oppositely polarized electron distributions. Atoms are attracted up to the 'van der Waals contact distance' after which repulsive forces dominate (Berg *et al.* 2007). The type and level of non-covalent interactions in a gel or food system depend on the protein concentration, the amino acid composition and protein folding, the ionic strength and pH of the environment and the level of denaturation after heating (Alleoni 2006).

## 2.2.2 Covalent cross-links

Under relatively mild conditions, SH groups and SS bonds can be involved in intramolecular and intermolecular **SH oxidation** and **SH-SS exchange reactions** (Figure 2.1). In the presence of a hydrogen acceptor, two free SH groups can oxidize and jointly form a SS bond (*ca.* 60 kJ/mol) (Singh and Whitesides 1993). SH-SS exchange reactions are initiated by a nucleophilic attack of a free thiolate ($S^-$) anion on the sulfur atom of an adjacent SS bond (Rombouts *et al.* 2012b). In water, SH groups ($pK_a$ 8.3) are more reactive under mild alkaline conditions than at neutral pH (Visschers and de Jongh 2005). Cross-linking of proteins increases their MW and often decreases their solubility. In some cases, protein cross-linking can be indirectly observed by the resultant loss of extractability in media containing sodium dodecyl sulfate (SDS) which disrupts non-covalent interactions. Reducing agents can break SS bonds (Singh and Whitesides 1993). Addition of oxidants and reducing agents to proteins before heating respectively hinders and facilitates polymerization during heating by the decrease and increase in accessible free SH groups (Lagrain *et al.* 2006). Furthermore, different types of enzymes can directly or indirectly affect SS bond formation (Joye *et al.* 2009a, b).

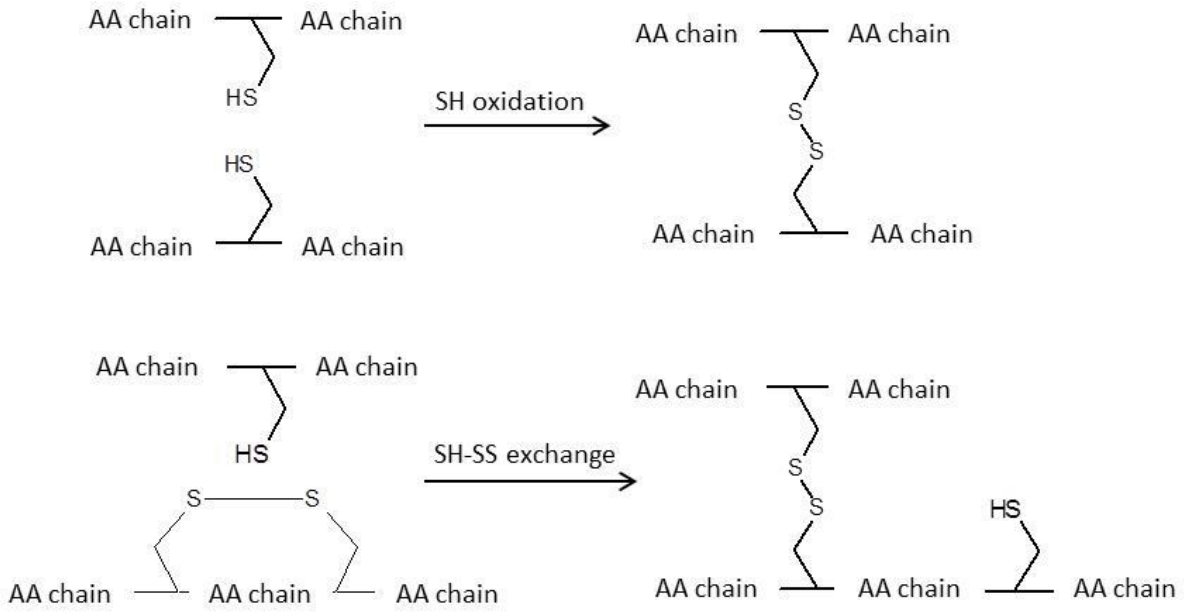



**Figure 2.1: Sulfhydryl (SH) oxidation reaction between two cysteine residues and a SH-disulfide (SS) exchange reaction between a cysteine and cystine residue of amino acid (AA) chains.**

At elevated temperatures and/or alkaline conditions **β-elimination reactions** of SS bonds induce the formation of free SH groups and DHA residues (Figure 2.1). Besides SS bonds, cysteine, serine and threonine residues can also be consumed in β-elimination reactions. However, their reactivity is much lower (about 3-7% of the rate of SS bonds) (Whitaker and Feeney 1983). Hereafter, **DHA** can react irreversibly by Michael addition with side chains of different amino acids such as cysteine, lysine, ornithine (originating from arginine) and histidine. Most frequently, DHA reacts with the

thiolate anion of cysteine and the unprotonated ε-amino group of lysine to lanthionine (LAN) and lysinoalanine (LAL) respectively (Figure 2.1) (Friedman 1999). LAN ($pK_a$ of cysteine is 8.3) can be formed at less alkaline conditions than LAL ($pK_a$ of lysine is 10.8) (Rombouts *et al.* 2012b). Alkaline treatment of proteins also has adverse effects on digestibility and nutritional values given the loss of lysine (Whitaker and Feeney 1983; Friedman 1999). LAL is toxic for rats but neither mutagenic nor carcinogenic (Friedman 1999; Corpet *et al.* 2008). The free SH groups formed during β-elimination reactions can be further involved in SS bond formation and reshuffling.

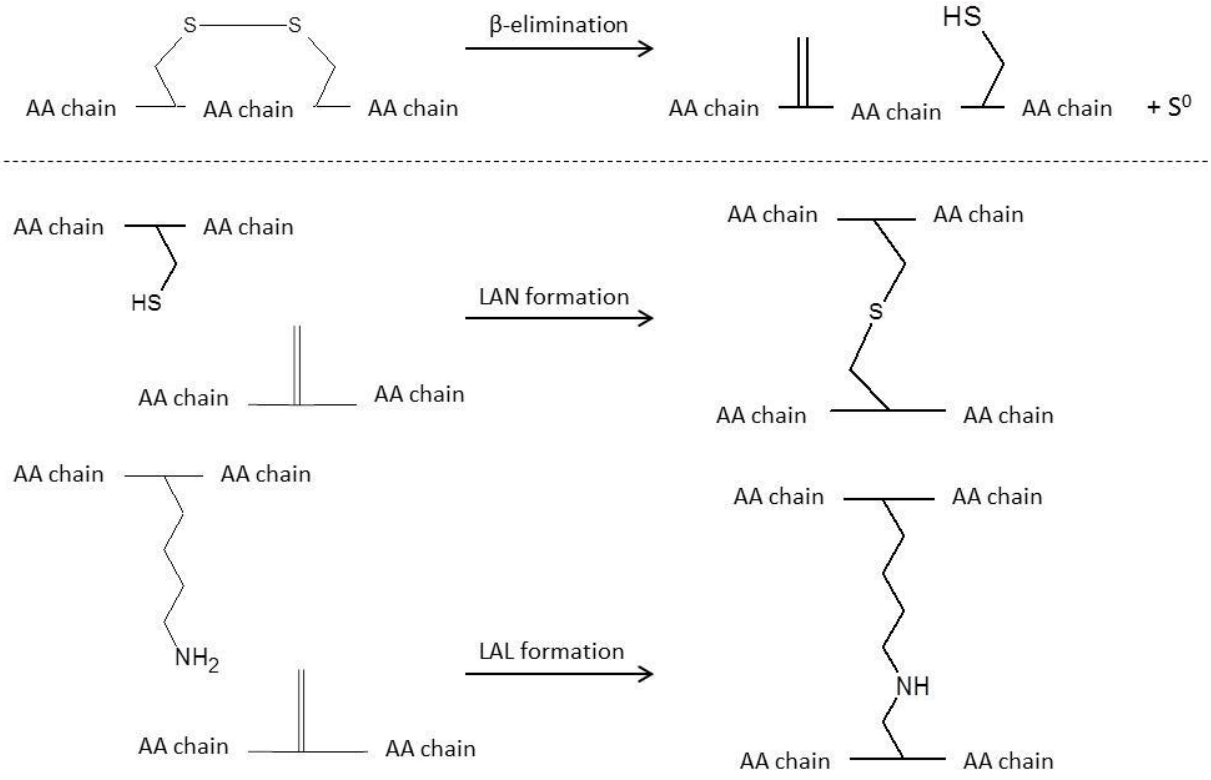



**Figure 2.2: β-Elimination reaction of cystine with formation of a dehydroalanine (DHA) and a cysteine residue, and formation of lanthionine (LAN) and lysinoalanine (LAL) by reaction of DHA with respectively cysteine or lysine in amino acid (AA) chains.**

**Isopeptide cross-links** between the ε-amino groups of lysine and the β- or γ-carboxamide groups of asparagine (Figure 2.3) or glutamine, respectively, are formed by severe heat treatment under dry conditions [*e.g.* 5-10% moisture content at 130 °C (Rombouts *et al.* 2011)] at neutral pH with loss of ammonia. Isopeptide bond formation is also catalyzed by the enzyme transglutaminase (EC 2.3.2.13) (Rombouts *et al.* 2012b). This enzyme can be used for controlled cross-linking of proteins without heating. This is *e.g*. done to assemble pieces of fish or meat (Belitz *et al.* 2009). Condensation reactions between the ε-amino groups of lysine and the β- or γ-carboxyl groups of aspartic or glutamic acid, respectively, are also possible. Such reactions release water instead of ammonia (Belitz *et al.* 2009). Isopeptide bond formation decreases in the presence of reducing sugars probably due to competition with Maillard reactions (Singh 1991).

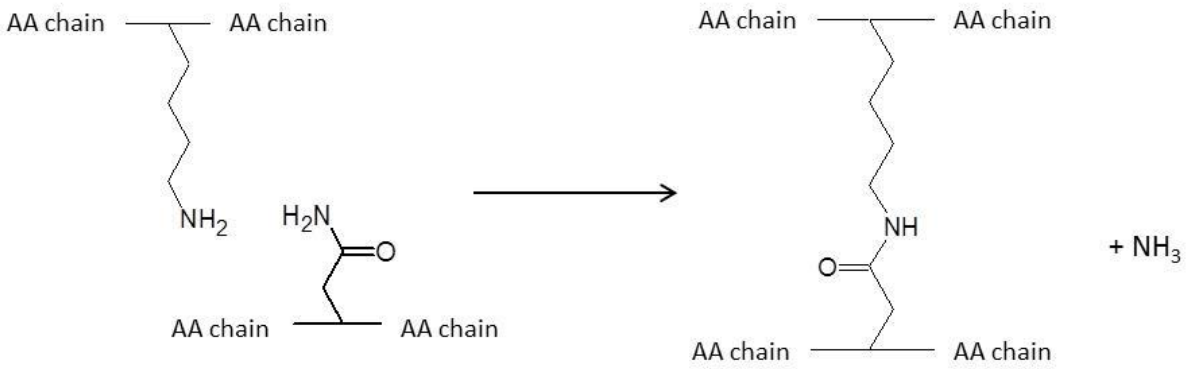



**Figure 2.3: Formation of an isopeptide cross-link between a lysine and an asparagine residue between two amino acid (AA) chains with loss of ammonia.**

**Dityrosine** and **trityrosine cross-links** are sometimes present in native proteins. They can result from oxidation of tyrosine residues in the presence of hydrogen peroxide and peroxidase (EC 1.11.1.7) (Singh 1991). The enzyme laccase (EC 1.10.3.2) can form isodityrosine and to a smaller extent dityrosine crosslinks by a radical mechanism (Figure 2.4) (Steffensen *et al.* 2008). Furthermore, tyrosine residues can convert into *o*-quinones in the presence of molecular oxygen and the enzyme tyrosinase (EC 1.14.18.1). These structures can then non-enzymatically react with reactive groups of *e.g.* lysine or cysteine (Rombouts *et al.* 2012b).

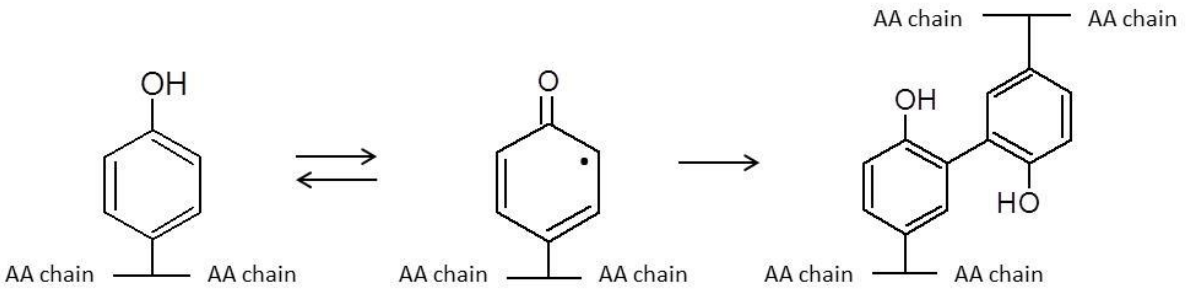



**Figure 2.4: Formation of dityrosine cross-links from tyrosine residues in two amino acid (AA) chains by a radical mechanism.**

Finally, different conditions (high temperature, relatively dry and/or alkaline conditions) can induce **Maillard reactions**, a complex cascade of reactions with various end products. It is typically initiated by condensation of an amine group with a carbonyl group of a reducing sugar or a lipid side product (Belitz *et al.* 2009). Maillard products impact flavor, aroma, color and nutritional characteristics *inter alia* at the crust of bread, cake and pastries (Rombouts *et al.* 2012b).

## 2.3 Network formation between proteins of one source

### 2.3.1 Wheat

#### 2.3.1.1 Network formation in wheat dough

In wheat dough, glutenin and gliadin mainly contribute to its elasticity and viscosity, respectively (Delcour and Hoseney 2010). A model of the gluten network visualizes gliadin and LMW-GS as spheres which interact with linear HMW-GS polymers (Figure 2.5.A). Dough making causes polymer alignment favoring end-to-end polymerization of HMW-GS (Belton 1999).

Although cysteine is a minor amino acid in gluten (*ca.* 2%), it is of great importance for the structure and functionality of wheat flour dough. Indeed, dough weakening or strengthening is observed upon addition of SS reducing and oxidizing agents, respectively (Wieser 2007). Gluten treated with reducing agents forms a viscous liquid rather than a three-dimensional network (Kontogiorgos *et al.* 2016). The SS status in glutenin can change from grain to end product. Its HMW-GS, B- and C-LMW-GS each contain at least two free SH groups which can act as chain extenders. D-LMW-GS only contain one free SH group and can act as chain terminators. Some SS bonds between cysteine residues of HMW-GS and LMW-GS have been detected (Lutz *et al.* 2012). Probably, HMW-GS form a backbone by end-to-end cross-links to which linear polymers of LMW-GS are attached (Figure 2.5.B). Shewry *et al.* (2001) have suggested that glutenin consists of a backbone of HMW-GS which has branched LMW-GS and that gliadin interacts non-covalently with glutenin and thereby contributes to the viscosity of gluten (Figure 2.5.C). Gliadin weakens the interactions between glutenin chains and plasticizes the gluten structure. The ratio of gliadin to glutenin determines the balance between elasticity and viscosity (Veraverbeke and Delcour 2002). The stability of intramolecular SS bonds in gliadin prevents them from becoming involved in SH-SS exchange reactions (Shewry *et al.* 2001). However, Lutz *et al.* (2012) have demonstrated the presence of some SS bonds between α-, γ-gliadins and LMW-GS. As less than 0.1% of the tyrosine residues are cross-linked in wheat dough, it is assumed that these are of minor importance (Hanft and Koehler 2005).

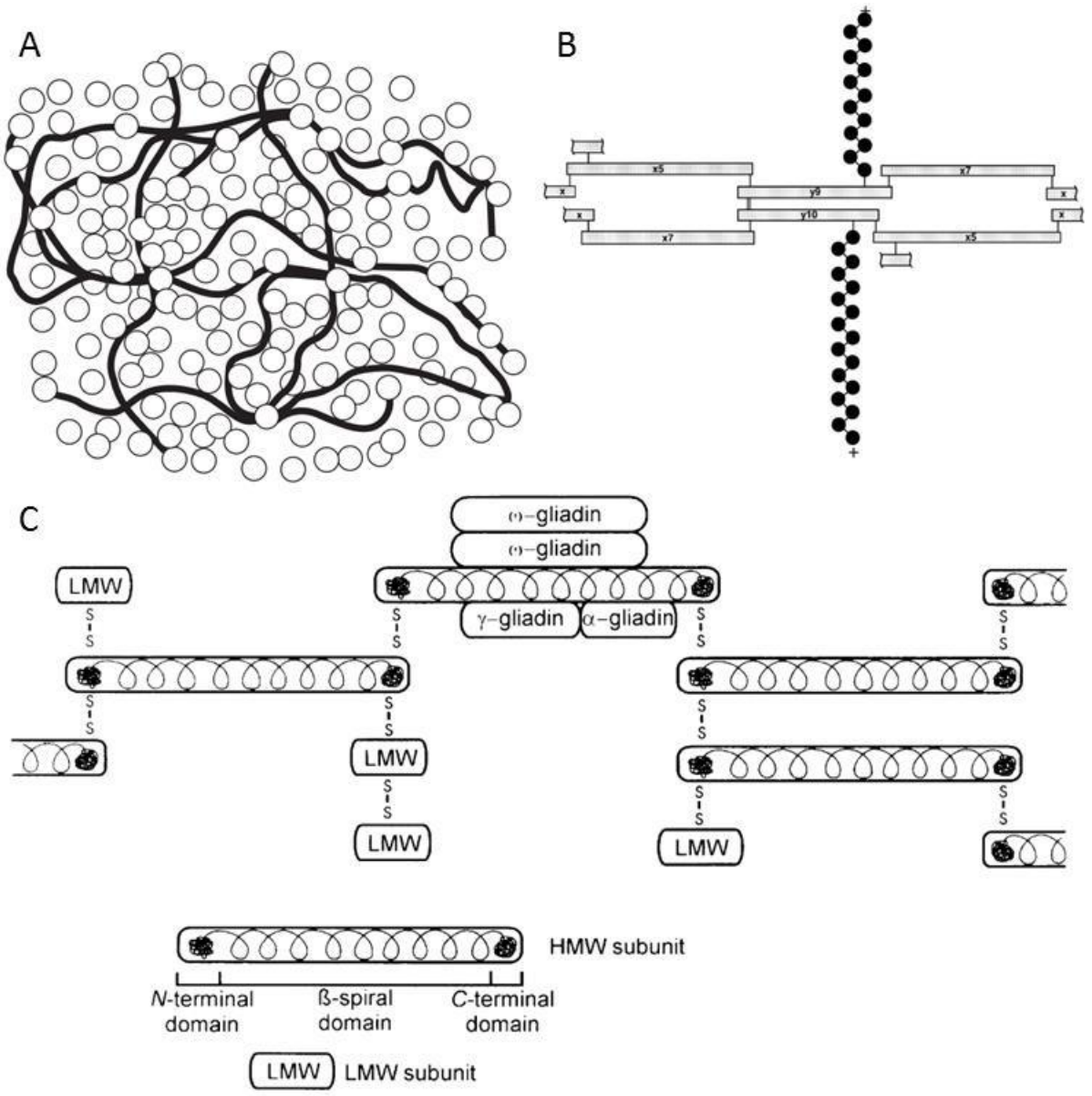



**Figure 2.5: Schematic models of gluten (A) with high molecular weight (HMW) glutenin subunits (GS) as linear polymers and other gluten proteins as spheres [not shown are the disulfide (SS) bonds present] (Belton 1999), the SS structure of glutenin (B) characterized by a backbone of x- and y-type HMW-GS (□) and linear low molecular weight (LMW) GS (●) chains (Wieser 2007) and the interactions between gluten proteins (C) where gliadin is non-covalently incorporated in a SS bound glutenin network (Shewry *et al.* 2001).**

The repetitive domains in gluten proteins are rich in glutamine which are prone to form hydrogen bonds (Delcour and Hoseney 2010). When all SS bonds in gluten are reduced, gluten forms a gel during heating which is stabilized by hydrogen bonds probably between intermolecular β-sheet structures (Dahesh *et al.* 2016; Kontogiorgos *et al.* 2016). Addition of urea or deuterium oxide weakens and strengthens gluten dough, respectively (Jankiewi and Pomeranz 1965; Tkachuk and Hlynka 1968). Next to SS bonds, hydrogen bonds largely impact the elasticity of the gluten network. Belton (1999) developed a model which suggests that mainly HMW-GS mutually associate through intermolecular hydrogen bonds. When water is added, proteins are hydrated and a balance between

solvent-protein 'loops' and protein-protein 'trains' interactions develops (Figure 2.6.A1 to 2.6.A3) (Belton 1999). At 40% moisture content, wheat dough has a compact protein structure which allows interprotein network formation without excess water in 'loops' (Huschka *et al.* 2012). The 'train' regions are formed by intermolecular β-sheet formation. Stretching extends the 'loops' in wheat dough of high moisture content, with increased formation of β-sheet structures at the expense of β-turns, and, eventually, proteins slide over each other (Figure 2.6.B1 to 2.6.B3). After stretching, the structure relaxes and the balance between loops and trains is restored (Belton 1999; Wellner *et al.* 2005). The conversion of β-turn into β-sheet structures during extension of gluten and the subsequent conversion of β-sheet into β-turn structures during relaxation confirm this model. However, repeated deformation increases the complexity of the gluten structure and the model could no longer be applied. (Wellner *et al.* 2005). Continued energy input can eventually destroy the gluten network (Delcour *et al.* 2012).

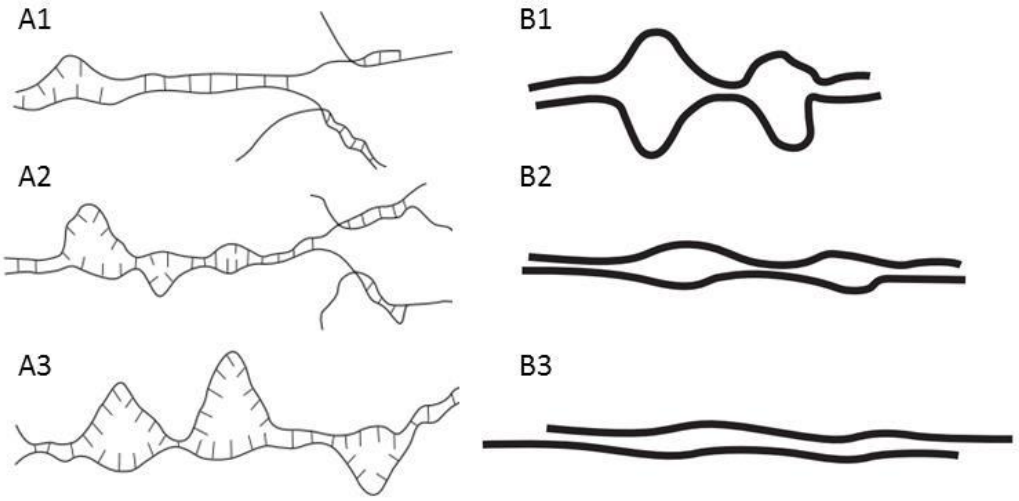



**Figure 2.6: Impact of hydration on the 'loop' and 'train' behavior of high molecular weight glutenin subunits (A) with low (A1), intermediate (A2) and high (A3) levels of hydration decreasing the amount of interchain bonds and the impact of extending this network (B) with small (B1), intermediate (B2) and large (B3) deformations breaking interchain hydrogen bonds (Belton 1999).**

Next to hydrogen bonds, hydrophobic interactions between nonpolar side chains are the most important non-covalent interactions in wheat dough (Bonomi *et al.* 2013). Soft wheat flour dough contains proteins with more low-affinity surface hydrophobic patches than hard wheat flour dough. Its proteins have less hydrophobic patches but these have higher affinity for non-polar residues (Huschka *et al.* 2012). In weak dough, gluten proteins are more prone to structural collapse and formation of β-sheet structures (Jazaeri *et al.* 2015). Jazaeri et al. (2015) suggested that β-sheets are formed to minimize the contact of water with hydrophobic patches at the surface of gluten proteins.

Although the level of ionic amino acids in gluten proteins is low, ionic interactions impact gluten network formation and dough development. The pI of gluten is about 7.5 (Mita *et al.* 1977). In flour-

water systems (pH around 6.1), gluten is positively charged. Lowering wheat dough pH with organic acids increases repulsion between positively charged gluten proteins and decreases mixing time and wheat dough strength (Galal *et al.* 1978). However, inclusion of salt in wheat dough recipes increases dough development time and, upon further mixing, dough strength and stability (Galal *et al.* 1978; Van Steertegem *et al.* 2013). Salt shields charges on gluten proteins (Galal *et al.* 1978).

Even though wheat albumin and globulins coexist with wheat gluten, little is known about their impact on network formation in wheat-based products. It is of note that Peruffo *et al*. (1996) identified SS bonds between wheat β-amylase and LMW-GS.

#### 2.3.1.2 Network formation during heating

During heating (above 45 °C), hydrogen bonds break and structural changes in gluten occur which increase its surface hydrophobicity (Guerrieri *et al.* 1996). Hydrophobic bonds differ from other bonds in the sense that their strength increases with temperature. In fact, they can provide additional stability during baking processes (Wieser 2007). Heating above 90 °C strongly decreases gluten extractability in acetic acid containing medium with formation of SS bond based aggregates (Guerrieri *et al.* 1996). Between 50 °C and 130 °C, cross-links are formed in gluten. These increase its MW and decrease its extractability (Domenek *et al.* 2002; Lagrain *et al.* 2010). In bread and pasta, glutenin polymerizes first while α- and γ-type gliadins are incorporated in the protein network at higher temperatures (Lagrain *et al.* 2008b; Bruneel *et al.* 2011). In gluten-starch model bread baking, glutenin polymerizes mainly through SH oxidation reactions below 90 °C, while α- and γ-gliadins become involved in SH-SS exchange reactions mainly at temperatures exceeding 90 °C. The rate of extractability loss during heat treatment of gluten suspensions at 95 °C is higher for γ-gliadin than for α-gliadin (Lagrain *et al.* 2008b). Starch does not impact the kinetics of their polymerization (Lagrain *et al.* 2008a). The importance of SS bond formation in protein networks in bread, cookies and pasta has also been demonstrated in experimental approaches involving the use of oxidizing, reducing and SH-blocking agents (Lagrain *et al.* 2008a; Pareyt *et al.* 2010; Bruneel *et al.* 2011; Bruneel *et al.* 2016).

Heating for 18 h at 110 °C forms covalent cross-links in gluten other than SS bonds (Guerrieri *et al.* 1996). Tilley *et al.* (2001) found higher levels of dityrosine cross-links in baked bread than in dough. At higher temperature and preferably alkaline pH, β-elimination reactions of intramolecular SS bonds in gliadin can release SH groups and DHA residues, which can then become involved in SH oxidation, SH-SS exchange and Michael addition reactions. Gliadin cross-linking at 110 °C and neutral or mild alkaline pH mainly results from intermolecular SS bond formation (Lagrain *et al.* 2011). In addition, hydrothermal treatment of gluten at 130 °C and pH 8.0 leads to the formation of LAN residues, while at pH 13.0 both LAN and LAL residues are formed (Rombouts *et al.* 2010). Both residues are also

found in pretzel proteins after alkaline dipping and baking (Rombouts *et al.* 2012a). Next to LAN and LAL, 3-methyl-LAN and 3-methyl-LAL residues which originate from various precursors (cystine, cysteine, lysine, serine and threonine) have been identified in gliadin heated (4 h, 130 °C) in water (Rombouts *et al.* 2015). Furthermore, isopeptide bonds have been detected in gluten heated (24 h, 130 °C) at low moisture content (7.4%) (Rombouts *et al.* 2011). Above 130 °C, gluten polymers can undergo thermal degradation events. Cooling of heat-treated gluten favors the formation of low energy interactions such as hydrogen bonds (Lagrain *et al.* 2010).

### 2.3.2 Hen eggs

Heating of whole eggs, egg white or egg yolk results in protein network formation and yields gels which entrap water. The water binding capacity, elasticity, transparency and/or hardness of these gels depend on the type and degree of protein-protein interactions which themselves depend on the number of accessible functional protein sites (Alleoni 2006).

During **egg white** heating, ovotransferrin is the first protein to denature. This protein starts egg white gelling (Section 1.3.1) when above a critical concentration (Broersen *et al.* 2006). Heat-induced (partial) denaturation of ovalbumin, ovotransferrin and lysozyme increases their levels of intermolecular β-sheets and enhances the accessibility of SH groups and SS bonds. SS cross-links are not necessary for egg white gelation but can enhance gel stability, elasticity and strength. The transformation of ovalbumin into the more heat-stable S-ovalbumin postpones its denaturation and therefore network formation (Mine 1995). Ovomucin and ovomucoid are the only proteins which do not participate in egg white gelling (Gossett *et al.* 1984). The strength of egg white gels increases in a pH range from pH 6.4 to 9.6 and with the concentration of salt (Holt *et al.* 1984). However, at pH values far from the pI, electrostatic repulsion between egg proteins obstructs network formation and gelling (Mine *et al.* 1990). Upon cooling, the contribution of hydrogen bonds to the strength of the protein network again increases (Mine 1995). In addition, boiling egg white induces DHA formation and, in the presence of reducing sugars, Maillard reactions (Lassé *et al.* 2015). Boiling egg white for two hours also forms LAN and LAL residues (Wouters *et al.* 2016). The level of LAL residues increases with storage time (higher pH) of raw eggs (Friedman 1999).

Even though **ovalbumin** contains four free SH groups, hydrophobic interactions are the driving force for its aggregation. SS bond formation impacts network formation and aggregate morphology but does not necessarily increase gel strength. In contrast, SS bonds reduce the rotational freedom of hydrophobic patches in a gel network to rearrange themselves to increase gel strength (Broersen *et al.* 2006). More free SH groups are consumed in SH oxidation reactions during heating at pH 8.8 than at pH 7.6 (similar to the pH of aged and fresh egg white respectively) (Van der Plancken *et al.* 2006).

Harder ovalbumin gels are formed at higher pH and higher ionic strength (Weijers *et al.* 2002). In conclusion, both non-covalent and SS bonds contribute to the gel network of ovalbumin. Their relative contribution depends on the pH, ionic strength and heating conditions (Mine 2002).

The compact structure of **lysozyme** is stabilized by four intramolecular SS bonds (Section 1.3.1.6). Reducing agents and/or the occurrence of SH-SS exchange reactions (upon heating, in the presence of SH groups) which involve these intramolecular SS bonds can destabilize the lysozyme structure, expose its hydrophobic groups, and abolish its enzyme activity. Lysozyme gels are formed by non-covalent interactions but SS bonds can stabilize the gel network (Li-Chan and Nakai 1991). In egg white, lysozyme is not enzymatically active when heated at 60 °C even if this temperature is far below that at which it denatures (Huopalathi *et al.* 2007). Mixtures of ovalbumin and lysozyme form stronger gels at neutral pH than those produced from their isolated proteins. This synergistic effect is ascribed to attractive ionic interactions between both proteins (Arntfield and Bernatsky 1993).

Apovitellenin from LDL and livetin, both of which are egg yolk plasma proteins, denature at relatively low temperatures (Kiosseoglou 2003). When **egg yolk** is heated, LDL starts denaturing at 70 °C and gelling at 75 °C (Tsutsui 1988) together with α- and γ- livetin while the other egg yolk proteins do not participate in the process. The structure of egg yolk granules cannot prevent α-HDL from denaturing but it prevents aggregation and gelling as the phosphocalcic bridges limit the extent to which structural changes occur (Anton *et al.* 2000).

Furthermore, different egg fractions can show **synergistic** effects during gelling. Heat-induced gels made of whole egg are of higher strength than gels made from egg white or egg yolk. The MW of aggregates formed by heating (70 °C, 2 min) egg white or yolk also differ from those in heated whole egg (Raikos *et al.* 2007).

### 2.3.3 Soy

The mechanism of heat-induced **soy protein** gel formation and the appearance of the resulting gels is affected by pH and salt concentration (Puppo and Anon 1998; Renkema *et al.* 2000). Fine-stranded gels formed at pH 7.6 are softer than coarse gels formed at pH 3.8 (Renkema *et al.* 2000). β-Conglycinin plays a much smaller role in gel formation of soy protein isolate at pH 7.6 than at pH 3.8 (Renkema *et al.* 2000; Renkema *et al.* 2001). Acidic gels are mainly stabilized by non-covalent interactions while in alkaline gels SS bonds also contribute (Puppo and Anon 1998). Heat-treatment of soy proteins at alkaline pH can even induce the formation of LAL residues (Friedman 1999).

During heating at 100 °C, **β-conglycinin** forms aggregates that are soluble in salt containing buffer of neutral pH. During heating, the subunits of β-conglycinin dissociate and aggregate through

hydrophobic interactions. While the hydrophobic protein patches are gathered in a hydrophobic core, the carbohydrate groups linked to β-conglycinin subunits and charged hydrophilic groups are located at the protein surface. This prevents the monomers from approaching each other (Guo *et al.* 2012).

During heating at 100 °C and neutral pH, **glycinin** forms both soluble and insoluble aggregates. Glycinin contains fewer carbohydrate groups than β-conglycinin and the repulsive barrier for thermal aggregation of the former is lower than that of the latter (Guo *et al.* 2012). Glycinin denatures and forms gels at higher temperatures than does β-conglycinin. At the same pH (pH 3.8 or 7.6) and protein concentration, gels of glycinin are firmer than those of β-conglycinin (Renkema *et al.* 2001). At pH 7.6 but not at pH 3.8, the SS bond connecting acid and basic polypeptides of glycinin breaks during heating (Lakemond *et al.* 2000a). In heat-induced glycinin gels, basic polypeptides are predominantly incorporated in the gel network at pH 7.6, while at pH 3.8 all glycinin subunits contribute (Lakemond *et al.* 2003).

At pH 3.8, **blends of glycinin and β-conglycinin** result in gels which are more elastic than expected based on data for the individual proteins (Renkema *et al.* 2001). When mixtures of β-conglycinin and glycinin are heated (100 °C) at neutral pH, the formed aggregates are smaller than those from separated glycinin. β-Conglycinin binds easily through hydrophobic interactions and increases the solubility of mixed aggregates by exposing hydrophilic groups. This restricts further aggregation and leads to smaller soluble aggregates than does glycinin (Guo *et al.* 2012).

### 2.3.4 Bovine whey

Heat-induced gelation of **whey proteins** is dominated by non-covalent interactions and fine-tuned by SS bonds (Havea *et al.* 2004). Denaturation of α-lactalbumin increases the viscosity of whey protein concentrate dispersions while β-lactoglobulin induces gelling. The thermostability of whey proteins is enhanced by low pH while their denaturation and gelling is enhanced by high pH (Boye *et al.* 1995). Most animal proteins (*e.g*. BSA and α-lactalbumin) are more susceptible to LAL residue formation than plant (*e.g*. soy and wheat) proteins (Singh 1991). Whey proteins contain relatively high concentrations of cystine and lysine residues which are precursors for LAL residues. Such residues occur in pasteurized milk and whey protein isolate (Friedman 1999).

While β-lactoglobulin and BSA aggregate when heated alone (Matsudomi *et al.* 1991), **α-lactalbumin** does not (Calvo *et al.* 1993; Schokker *et al.* 1999).

During heating, **β-lactoglobulin** dimers dissociate at neutral pH into monomers and unfold with exposure of hydrophobic amino acid residues and its free SH group. Mainly above 78 °C, the latter

initiates polymerization with formation of insoluble aggregates (Sava *et al.* 2005), mostly by SH-SS exchange and to a lesser extent by SH oxidation reactions (Schokker *et al.* 1999). Point mutation of the free cysteine of β-lactoglobulin into a serine obstructs heat-induced covalent network formation (Jayat *et al.* 2004). Finally, the strength of heat-induced β-lactoglobulin gels increases with addition of low concentrations of salt which demonstrates the role of non-covalent interactions.

Salt does not impact the strength of gels made with **BSA**. Reducing agents lower the strength of such gels (Matsudomi *et al.* 1991) but hydrophobic interactions remain more important to initiate BSA gelling than SS cross-linking (Boye *et al.* 1996). Furthermore, the conformation of BSA changes during heating and when varying the pH with formation of intermolecular β-sheet structures (Boye *et al.* 1996; Militello *et al.* 2004). LAN, LAL, 3-methyl-LAN and 3-methyl-LAL residues have been identified in BSA heated for 4 h at 130 °C in water (Rombouts *et al.* 2015).

A **mixture** of β-lactoglobulin and α-lactalbumin gels at low protein concentration while the isolated proteins do not. Together, these proteins undergo SH-SS exchange reactions which stabilize the gel network (Matsudomi *et al.* 1992). A mixture of BSA (containing one free SH group) and α-lactalbumin (containing no free SH groups) forms heat-induced SS cross-linked polymers made up by the separate proteins and polymers of both (Havea *et al.* 2000). The higher gel strength of a gel from a β-lactoglobulin/BSA mixture than those of gels of the separated proteins has been attributed to the increased extent of SH oxidation during heating (Matsudomi *et al.* 1994). According to Gezimati *et al.* (1997) heat-induced gelation between whey proteins depends on their thermal transition temperatures, the availability of free SH groups and the ability to form non-covalent interprotein aggregates prior to SS bond formation.

## 2.4 Network formation between different types of proteins

Interactions and reactions between proteins of different sources can impact their behavior in food systems. Erickson *et al.* (2012) referred to this as a co-protein effect. Some examples for different proteins from the same source have been mentioned above. Co-protein effects between proteins of different sources are discussed here.

Addition of whey to egg white proteins enhances the strength of heat-induced gels. More hydrophobic groups are exposed and β-sheet structures formed in gels from mixtures of proteins than in those from isolated proteins (Ngarize *et al.* 2004; Ngarize *et al.* 2005). Ovalbumin enhances α-lactalbumin aggregation during heating by SH-SS exchange reactions (Sun and Hayakawa 2001). Also, zein/casein-based resins are of higher mechanical strength than zein-based resins and have more intermolecular β-sheets structures than expected based on observations for both isolated proteins

(Erickson *et al.* 2014). Addition of whey, soy or blood protein isolate, respectively, weakens, enhances or does not impact the strength of collagen gels. However, blood protein isolate enhances the formation of fibrillary structures and hence changes the aggregation mechanism. Gluten does not interact with collagen. Instead, two phases are formed in heat-induced gluten/collagen gels (Oechsle *et al.* 2015). At pH 8.0, heat-induced polymerization of a BSA/gluten mixture is similar to that of isolated gluten suggesting that the protein network formation of gluten dominates that of BSA. Gluten decelerates cross-linking of BSA and increases its denaturation temperature (Rombouts *et al.* 2012c). Wouters *et al.* (2016) found synergistic effects in heat-induced polymerization and gel firmness between gluten and egg white but not between gluten and casein.

While many examples of co-protein effects have been reported, the underlying mechanisms are not clear. Interprotein interactions depend *inter alia* on protein size, shape, pI, the structural distribution of charged, hydrophobic and other amino acids, as well as on the solvent (Foegeding and Davis 2011). As proteins differ in solubility, different protein types can be present in various phases. Polyakov *et al.* (1997) noticed phase separation between proteins with differences in hydrophilic character in aqueous solutions and referred to this phenomenon as protein thermodynamic incompatibility. Phase separation can promote interactions between proteins in the same phase (Polyakov *et al.* 1997). For example, mixtures of whey and soy proteins phase separate during heat-induced gelling probably due to large differences in MW and solubility in water (Comfort and Howell 2002). However, the impact of thermodynamic incompatibility on the occurrence of co-protein effects remains to be investigated.

In wheat flour dough making, inclusion of whole egg increases the time needed to develop it, its strength and its stability upon further mixing. At the pH of dough, negatively charged egg proteins can shield positively charged gluten proteins. Egg white increases wheat dough development time, strength and stability while egg yolk reduces these dough properties. These observations have been linked to ionic interactions and the presence of egg yolk lipids (Van Steertegem *et al.* 2013). Whey proteins also increase dough development time and wheat dough stability (Ammar *et al.* 2011). Inclusion of soy flour weakens wheat dough and increases the extractability of gluten proteins in SDS containing medium. Soy proteins interact with wheat proteins through non-covalent interactions and SS bonds during mixing and dough resting. Some gluten proteins are released from the gluten network as a result of SH-SS exchange reactions with soy proteins (Pérez *et al.* 2008). Heat-treatment of soy flour or partial reduction and oxidation of wheat/soy doughs increases covalent network formation (Maforimbo *et al.* 2008).

Furthermore, use of whey, soy or egg proteins impacts the quality of cereal-based food products including bread (Kenny *et al.* 2000; Ribotta *et al.* 2005; Nilufer *et al.* 2008), cake (Lee *et al.* 1993; Raeker and Johnson 1995; Ratnayake *et al.* 2012), cookies (Singh and Mohamed 2007), tortillas (Suhendro *et al.* 1993), pasta (Alamprese *et al.* 2005a; Lamacchia *et al.* 2010) and noodles (Section 2.5.2). Wheat and globular proteins can impact each other's network formation but the underlying mechanisms are poorly understood. It is of interest to link specific protein characteristics to their functionality in complex mixtures with wheat proteins.

## 2.5 Protein network formation in wheat-based noodles

Wheat-based noodles are popular all over the world. In Asia, they are staple foods. Noodles are made from common wheat (*Triticum aestivum* L.) flour using either soft or hard common wheat cvs by dough sheeting. In comparison, pasta is produced from durum wheat (*T. turgidum* L. *var. durum*) semolina by dough extrusion. Besides wheat flour, water, salt and/or alkali are included in wheat noodle dough (Fu 2008). In addition, the recipes of some pasta and noodle (*e.g.* Wonton noodles) products include eggs to enhance nutritional value (Li *et al.* 2014), flavor (Alamprese *et al.* 2012) and/or quality. With the growing interest in protein-rich food systems, also other protein sources of high nutritional value are included in wheat noodle recipes which otherwise are lysine deficient. However, using different protein sources in wheat-based noodle recipes impacts noodle quality (Li *et al.* 2014).

### 2.5.1 Protein network formation during noodle processing

Noodle and pasta dough are less developed than bread dough due to the lower moisture content (30-38%) and energy input during mixing and sheeting or extruding (Icard-Vernière and Feillet 1999; Hou 2010). Mixing uniformly distributes and hydrates noodle constituents (Moss *et al.* 1987). It increases the molecular mobility of the gluten proteins and the amorphous part of starch (Figure 2.7) (Cuq *et al.* 2003), thereby increasing the solubility of glutenin in dilute acid or SDS containing medium (Icard-Vernière and Feillet 1999; Bruneel *et al.* 2011). During a first dough rest (10 to 30 min), water in the crumbly dough is further distributed and gluten proteins relax. During compounding, dough crumbs are sheeted several times at the same gap sizes. After each pass through the rolls, two dough sheets are combined (Hou 2010). Under compression, adjacent flour endosperm particles fuse and form a continuous protein matrix which embeds starch granules (Moss *et al.* 1987). Sheeting allows gluten to align and develop in its direction (Dexter *et al.* 1979) but does not change its extractability in SDS containing medium (Bruneel *et al.* 2011; Rombouts *et al.* 2014). During a second dough rest, moisture further distributes uniformly, the gluten structure again relaxes and bonds between noodle constituents are enhanced (Hou 2010). Both resting phases increase the continuity of the protein

network resulting in smoother dough sheets. During reduction sheeting, pasta or noodle dough is successively sheeted through rolls with reducing gap sizes. This allows further gluten development (Moss *et al.* 1987). When the final dough thickness is reached, dough sheets are cut into noodles with desired widths and lengths. In fresh noodles, the protein matrix only partially covers starch granules at the surface (Figure 2.8.A) (Dexter *et al.* 1979).

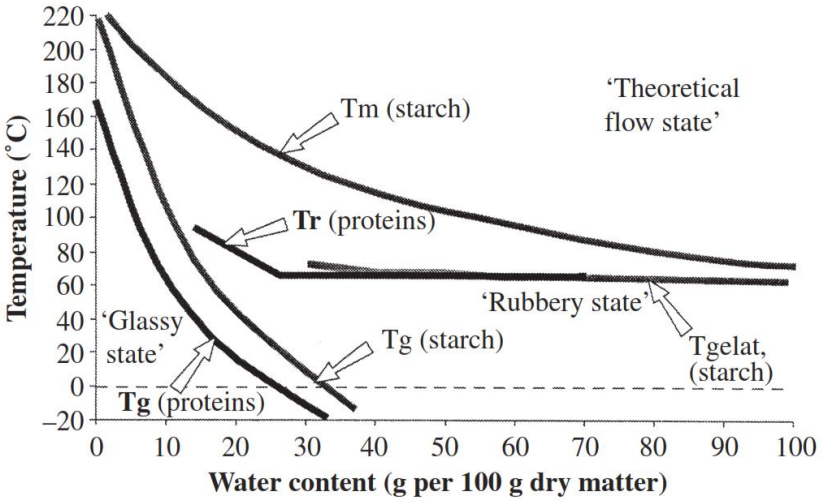



**Figure 2.7. 'Temperature-water content' state diagram of wheat flour proteins and starch with $T_g$ the glass transition temperature, $T_m$ the melting point, $T_{gelat}$ the gelatinization temperature of starch and $T_r$ the minimum temperature of protein thermosetting (Cuq *et al.* 2003).**

During cooking, water penetrates the noodle from surface to core (Kojima *et al.* 2001). It enhances protein polymerization and starch swelling and gelatinization (Petitot *et al.* 2009). Water indeed acts as plasticizer and is critical for protein polymerization and starch gelatinization (Cuq *et al.* 2003). During pasta cooking, glutenin polymerization is six times faster than incorporation of gliadin in the network (Bruneel *et al.* 2011). Bruneel *et al.* (2010) postulated that an optimum extent of protein network formation during drying and/or cooking is critical for durum wheat pasta quality. On the one hand, too much gliadin incorporation tightens the protein network, making it less flexible to cope with starch swelling during cooking and resulting in low pasta quality (Bruneel *et al.* 2016). On the other hand, pasta with insufficient protein polymerization during cooking lacks a continuous framework resulting in soft and sticky pasta (Resmini and Pagani 1983). In optimally and overcooked pasta, little if any cross-links other than SS bonds are present (Wagner *et al.* 2011). However, the inclusion of alkaline salts in noodle recipes induces the formation of LAN and LAL during cooking (Rombouts *et al.* 2014). Increasing the amount of cross-links by using transglutaminase increases dough strength and hardness of respectively fresh and cooked noodles. The impact of isopeptide cross-links is more pronounced in noodles made with flour of low than in noodles made with flour of high protein content (Wu and Corke 2005). Regretfully, the impact of non-covalent interactions on noodle processing and properties is not well described.

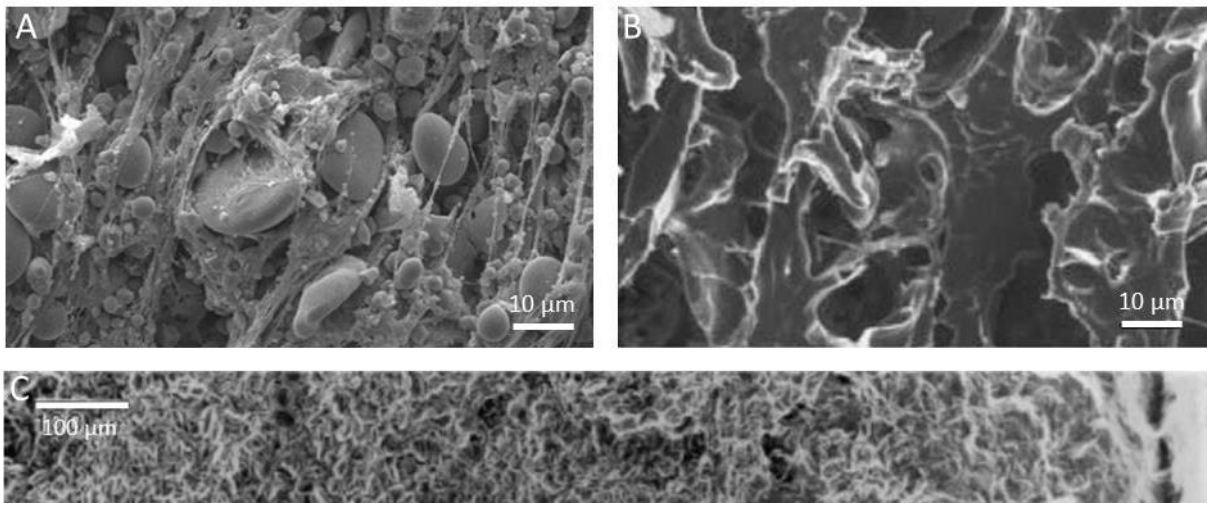



**Figure 2.8. Scanning electron microscopy photos of a cross-section of fresh noodles (A) (Li *et al.* 2012), the core of noodles cooked for 20 min (B) (Lai and Hwang 2004) and the internal structure of optimally cooked spaghetti (C) showing increasing extent of starch gelatinization from core (left) to surface (right) (Heneen and Brismar 2003).**

The rate of water uptake when cooking pasta to its optimum cooking time depends on its diffusion through the matrix (Maeda *et al.* 2009). During the early stages of the process, starch in the outer layers immediately absorbs water and then gelatinizes. Evidently, during the process, the degree of gelatinization decreases towards the noodle core (Figure 2.8.C) (Dexter *et al.* 1979). Gelatinization involves swelling of starch granules. They are partially disrupted and mainly amylose, *i.e.* unbranched starch, leaches out (Goesaert *et al.* 2005). The crystalline part of starch irreversibly loses it structure above its melting temperature (Cuq *et al.* 2003).

Noodles made with high protein flour have a dense and strong gluten network. Here, starch swelling and water imbibition are slower than in noodles made with low protein flour (Kojima *et al.* 2004). Prolonged heating increases the moisture content and the extent of starch gelatinization in the core until starch and protein can no longer be visually distinguished (Figure 2.8.B) (Lai and Hwang 2004).

### 2.5.2 Impact of different protein types on noodle quality

Fresh noodles should have a fine balance between dough elasticity and extensibility for optimum sheeting operations. Highly elastic gluten improves recoil of dough in the sheeting direction, which hinders the reduction of dough thickness during sheeting (Hou 2010). The quality of cooked noodles is typically evaluated in terms of color, symmetry, cooking quality (low water absorption and cooking loss) and texture (Oh *et al.* 1983). Also, a low optimal cooking time defined as the smallest time needed for the white noodle core to disappear or the starch to be gelatinized, is desired for all noodle types (Hou 2010). Due to the wide variety of noodle types and regional differences in textural preferences, noodle texture and therefore quality cannot be defined uniformly (Fu 2008; Hou 2010).

The tensile strength of fresh noodles linearly increases with increasing **gluten** incorporation (Park and Baik 2009). The optimal cooking time and brightness of noodles are respectively positively and negatively correlated with wheat protein content (Oh *et al.* 1985). Water imbibition during noodle cooking is lower when flour protein contents are higher and thereby increases optimal cooking time (Park and Baik 2009). Both wheat protein content and composition are related to the texture of white salted cooked noodles (Hou *et al.* 2013). While wheat albumin and globulin negatively impact noodle texture (Park *et al.* 2003; Hou *et al.* 2013), the LMW-GS and gliadin contents positively impact it (Hou *et al.* 2013). It has also been reported that the HMW-GS composition is related to the hardness of white salted noodles (Park *et al.* 2003). Park and Baik (2009) suggested that adding of extra gluten increases protein network formation in fresh noodles and reduces losses during cooking. Regardless of the cooking time, the tensile strength and hardness of cooked noodles increases with gluten concentration (Park and Baik 2009). In spite of the above, the impact of different Osborne fractions on protein network formation and their relation with noodle properties remain to be investigated.

Inclusion of **eggs** in their recipe improves the cooking quality of especially soft wheat flour noodles (Dalbon *et al.* 1996). The break load of cooked pasta increases with total egg content (Alamprese *et al.* 2009). Cooked whole egg noodles have less cooking loss, water uptake and a firmer texture and are more yellow than noodles from recipes with a total replacement of whole egg by egg substitutes (whey, wheat and soy isolates) (Khouryieh *et al.* 2006). A higher egg white to yolk ratio leads to increased strength of both fresh and cooked pasta. At the same time, it increases the protein and reduces the lipid contents of pasta (Alamprese *et al.* 2009). Furthermore, Shimoyamada *et al.* (2004) suggested that dry-heated egg white improves the texture and the sensorial properties of Chinese noodles through interaction with gelatinized starch rather than with gluten proteins. In spite of the above, the underlying mechanisms whereby and to what extent egg constituents impact noodle quality remain to be elucidated.

Also, replacing whole egg by **soy** protein isolate decreases cooking quality and noodle hardness. (Khouryieh *et al.* 2006). Substitution of whole egg by 20% minced **fish** decreases the firmness of cooked noodles (Setiady *et al.* 2007). Inclusion of minced **chicken** meat in wheat noodle recipes increases the firmness of both fresh and steamed noodles (Khare *et al.* 2015). Use of **buckwheat** in wheat noodle recipes increases the rate of water diffusion during cooking and lowers the optimal cooking time (Maeda *et al.* 2009). Even inclusion of spray-dried **blood plasma** (2.2% on dm) in soft wheat flour recipes increases the overall sensorial acceptability and texture of pasta made therefrom (Yousif *et al.* 2003). The chemical composition of egg substitutes seems to impact noodle quality more than their protein content (Khouryieh *et al.* 2006). However, the interplay between different

protein sources on protein network formation during noodle cooking and thereby the quality of noodles remains to be explored in detail.

## 2.6 Conclusion

Non-covalent (hydrogen, ionic and hydrophobic) interactions and the formation of SS bonds through SH oxidation and SH-SS exchange reactions dominate food protein network formation. Dityrosine and isopeptide cross-links can also be formed along with cross-links resulting from Maillard reactions, but they play a limited role in food systems and will not be studied here. At higher temperatures, β-elimination reactions transform SS bonds in free SH groups and DHA residues. The latter can react with cysteine or lysine residues to form LAN and LAL cross-links. As these reactions are favored at alkaline pH their impact on quality of most cereal-based food products is low.

Proteins in one food source can impact each other's network formation. Upon mixing with water, gliadin and glutenin can form a visco-elastic gluten network. Glutenin has been assumed to form an elastic backbone of HMW-GS linked by SS bonds. In this model for glutenin, LMW-GS are attached to this backbone by SS bonds and non-covalent interactions, while gliadin interacts non-covalently. During heating, gliadin is covalently incorporated in the glutenin network mainly through SH-SS exchange reactions. Furthermore, different proteins in egg, soy and whey impact each other's network formation and thereby gelling properties. For example, BSA and β-lactoglobulin can induce gelling of α-lactalbumin through SH-SS exchange reactions while isolated α-lactalbumin does not form gels.

Interactions and reactions between proteins of different food sources can also induce co-protein effects. Literature describes synergistic, antagonistic and no effects when mixing different protein sources in dough systems or in the production of heat-induced gels. However, the impact of different proteins on each other's denaturation and polymerization and the underlying mechanisms are insufficiently known. Especially the impact of different food proteins on heat-induced gluten network formation remains to be investigated and is relevant for many cereal-based food products.

In Chapter 3 of this dissertation, a method for studying covalent network formation of systems which contain various protein types will be developed. The possible impact of thermodynamic incompatibility on co-protein effects between gluten and globular proteins will be investigated in Chapter 4. In most cereal-based food products water or aqueous salt solutions are used as liquid phase in which gluten is insoluble while egg, soy and whey proteins are not. Here, polymerization of mixtures of gluten and globular proteins in water will be compared to that in a medium in which all protein types are soluble. The relation between protein characteristics and possible co-protein

effects in mixtures with gluten during heating in water will be studied in Chapter 5. The impact of different types of proteins (especially egg proteins) on protein network formation and properties of wheat-based noodles will be investigated in Chapters 6 and 7. Noodles will be made from a lean formula (flour, water and salt) thereby using a straightforward protocol with few processing steps (mixing, sheeting and slitting). Even if it has been demonstrated that the use of different types of proteins impacts the properties and quality of noodles, protein network formation during production and cooking has only been described for gluten.

# PART TWO

# Experimental work

## Chapter 3

**Non-size effects in size exclusion chromatography**

## Chapter 4

**Thermodynamic compatibility of proteins:
A case study in aqueous ethanol and water**

## Chapter 5

**Heat-induced polymerization of different food proteins
and mixtures thereof with wheat gluten in water**

## Chapter 6

**The role of wheat and egg constituents
on protein network formation in egg noodles**

## Chapter 7

**The impact of various model proteins
on protein network formation in noodles**

# Chapter 3

# Non-size effects in size exclusion chromatography

## 3.1 Introduction

Size exclusion (SE) chromatography is widely used in biology, biochemistry, (food) chemistry and pharmacology to purify proteins and to study their apparent MW (Ricker and Sandoval 1996) and aggregation (Carpenter *et al.* 2010). Ideally, SE high performance liquid chromatography (HPLC) elutes proteins in order of decreasing hydrodynamic volume, and thus size (Kopaciewicz and Regnier 1982). Therefore, it is a valuable tool to study heat-induced changes in molecular weight distributions for different proteins. However, hydrogen, ionic, hydrophobic and Van der Waals interactions between chromatographic resins and proteins can occur, sometimes even followed by protein conformational changes (Irvine 1987; Dubin and Principi 1989; Kamberi *et al.* 2004). Such non-ideal interactions lead to non-size effects including changes in elution time, chromatographic resolution, peak shape and even level of detected protein (Arakawa *et al.* 2010). Furthermore, they can weaken non-covalent interactions between proteins thereby hampering the quantification of non-covalently associated protein aggregates (Kamberi *et al.* 2004). In some cases, non-size effects are helpful. For instance, Ovalle (1995) separated proteins with similar MW but different pI on a SE column using a zwitterionic buffer. Either way, it is of the utmost importance to be well informed of potential non-size effects.

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This chapter is based on the following reference: Lambrecht M.A., Rombouts I., Van Kelst L., Delcour J.A. **2015**. Impact of extraction and elution media on non-size effects in size exclusion chromatography of proteins. Journal of Chromatography A, 1415: 100-107.

The level of interactions between proteins and resins depends on the resin type, the proteins involved and the mobile phase (Arakawa *et al.* 2010). Two types of SE resins can be distinguished namely (i) inorganic materials based on silica particles coated with a hydrophilic outer layer and (ii) organic polymeric cross-linked particles with a more hydrophobic character (Irvine 2003). In this study silica-based resins of well-defined pore-size distribution are used. These resins are rigid, stable towards a variety of mobile phases between pH 2 and 8 (Irvine 1997), and can easily be regenerated after exposure to denaturants (Ahmed and Modrek 1992). For uncharged (electrically neutral) solutes, distilled water is a suitable eluent (Aguilar 2004). For albumins and globulins, non-ideal interactions can be minimized by adding co-solvents like salts, organic modifiers, detergents or amino acids to the elution solvent (Kamberi *et al.* 2004; Arakawa *et al.* 2010). Nearly ideal SE chromatography can be executed by minimizing ionic and hydrophobic interactions which suggests minor influence of hydrogen and Van der Waals interactions (Irvine 1987).

The most common eluents can be divided in two types: inorganic buffers and mixtures of water with organic modifiers. For silica-based resins, inorganic buffers with an ionic strength between 0.1-0.5 M are generally used (Stulík *et al.* 2003). At low ionic strength, ionic interactions between resins and proteins occur (Kopaciewicz and Regnier 1982; Irvine and Shaw 1986). Ionized silanol residues ($pK_a$ 3.5-4.0) impart it with cation-exchange properties. They form an anionic field which interacts with proteins (Kopaciewicz and Regnier 1982; Ovalle 1995). The addition of salts decreases the ionic interactions between resin and proteins (Irvine 1987; Ovalle 1995; Kamberi *et al.* 2004). However, high ionic strength can induce hydrophobic interactions due to solvophobic (Kopaciewicz and Regnier 1982) or salting-out effects (Arakawa *et al.* 2010). Under these conditions even slightly hydrophobic coatings can interact with proteins. When the conformations of proteins in mixtures are unknown, denaturing agents like urea or guanidinium hydrochloride, or anionic surfactants like SDS are often added to the extraction medium. For denatured proteins, the hydrodynamic volume, which can be deduced from its elution time, correlates well with peptide chain length and protein MW (Montelaro *et al.* 1981; Mant *et al.* 1987; Irvine 1997). Acetonitrile is a popular organic modifier even though it can enhance ionic interactions and at concentrations exceeding 40% induce conformational changes in proteins (Gekko *et al.* 1998). Trifluoroacetic acid (0.05% or 0.1% w/v) is often added. It is ultraviolet (UV) transparent, can increase protein solubility and decrease non-ideal hydrophobic interactions (Irvine and Shaw 1986). It suppresses ionization of silanol residues (Lau *et al.* 1984; Irvine 1987) but some cation-exchange capacity at low pH can remain due to ion-pairing (Irvine and Shaw 1986; Irvine 1987). Combinations of co-solvents can impact the intended effect. For instance, sodium chloride and acetonitrile in the elution solvent counterbalance each other's non-size effects (Kamberi

*et al.* 2004). The combination of other co-solvents and their impact on non-size effects remain to be investigated.

SE-HPLC is *e.g.* also useful for evaluating changes in protein MW during food processing (Schofield *et al.* 1983), but the study of non-size effects in this context is more complex. Most food systems contain different protein types. In some food products, *e.g.* pastry, bread, noodles and pasta, wheat proteins can coexist with egg, soy or milk proteins. In contrast to albumins and globulins which have already been studied in the context of non-size effects, gluten proteins are mostly insoluble in water and salt solutions and non-size effects impacting these proteins remain to be investigated. To evaluate heat-induced changes in MW of cereal proteins, extraction is often performed in SDS containing media (Batey *et al.* 1991; Weegels *et al.* 1996; Thanhaeuser *et al.* 2014). It is unclear whether SDS and other co-solvents in extraction media impact non-size effects. Even though concentrations are low, co-solvents in extraction media can impact both protein conformation and column properties.

Against this background, we here optimized a SE-HPLC method for studying various protein types present in food. SDS containing media extract almost all proteins from cereals, milk, eggs and soy. Therefore, as a first objective, the impact of elution solvents on non-size effects between a silica-based resin and proteins extracted in SDS containing medium was investigated. The second objective was to study non-size effects for various combinations of extraction media and elution solvents.

## 3.2 Materials and methods

### 3.2.1 Materials

**Wheat** kernels (cv Paragon) from RAGT (Ickleton, United Kingdom) were conditioned to 16.0% moisture and milled with a Bühler (Uzwil, Switzerland) MLU-202 laboratory mill (Delcour *et al.* 1989) to obtain wheat flour with 13.7% protein on dm basis. **Gluten** (83.2% protein on dm) was isolated from flour with a dough ball method. Flour (98.6 g, 12.5% moisture) was mixed with 55.0 ml deionized water in a pin-mixer (National Manufacturing, Lincoln, NE, USA) for 150 sec to form an optimal dough as estimated by a mixograph [National Manufacturing, AACC I Approved Method 54-40.02 (AACC 1999)]. After a dough rest of 60 min, gluten was separated from dough by washing with deionized water (*ca.* 1 000 ml). The fraction above 38 µm is called gluten. It was then freeze dried and ground in a laboratory mill (IKA, Staufen, Germany) and passed through a 250 µm sieve. **Soy glycinin** (98.1% protein on dm) was extracted according to Liu *et al.* (2007) from soy flour (L.I. Frank, Twello, The Netherlands), freeze dried and ground in a laboratory mill (IKA). **BSA** (fraction V for biochemistry) was from Acros Organics (Geel, Belgium) and contained 98.2% protein on dm. **Hen egg**

**ovalbumin** (albumin chicken egg grade III) was purchased from Sigma-Aldrich (Bornem, Belgium) and contained 94.1% protein on dm. All chemicals were at least of analytical grade and from Sigma-Aldrich unless specified otherwise. Urea, dithiothreitol (DTT), disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International (Leuven, Belgium).

### 3.2.2 Protein content determination

Protein contents of gluten, glycinin, BSA and ovalbumin samples and their extracts were determined in triplicate using an adaptation of the AOAC Official Method 990.03 (AOAC 1995), with an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands). Conversion factors (5.7 for wheat flour and gluten; 6.25 for soy glycinin, BSA and ovalbumin) were used to calculate protein from nitrogen contents.

### 3.2.3 Size exclusion high performance liquid chromatography

Proteins (1.0 mg protein/ml extraction medium) from gluten, soy glycinin, BSA and ovalbumin samples were **extracted/dissolved** (60 min, room temperature) with different media: water, sodium phosphate medium (0.050 M; pH 7.6) with 0.4 M sodium chloride, hereafter referred to as salt medium, or sodium phosphate buffer (0.050 M; pH 6.8) containing (i) 2.0% (w/v) SDS, further referred to as SDS medium, (ii) 2.0% (w/v) SDS with 2.0 M urea, (iii) 2.0% (w/v) with 1.0% (w/v) DTT and (iv) 2.0% (w/v) SDS with 2.0 M urea and 1.0% (w/v) DTT (Table 3.1). Table 3.1 provides an overview of the used extraction/solubilizing media with their short names.

**Table 3.1: Overview of the used extraction/solubilizing media with their short names. SDS, sodium dodecyl sulfate and DTT, dithiothreitol.**

| Extraction medium | Short name |
|---|---|
| Water | Water |
| Sodium phosphate buffer (0.050 M; pH 7.6) containing 0.4 M sodium chloride | Salt medium |
| Sodium phosphate buffer (0.050 M; pH 6.8) containing 2.0% (w/v) SDS | SDS medium |
| SDS medium containing 2.0 M urea | SDS/urea medium |
| SDS medium containing 1.0% (w/v) DTT | SDS/DTT medium |
| SDS medium containing 2.0 M urea and 1.0% (w/v) DTT | SDS/urea/DTT medium |

When the extraction medium contained DTT, the extraction was performed under nitrogen atmosphere. All extractions were in triplicate and followed by centrifugation (10 000 *g*, 10 min) and filtration (Millex-HP, 0.45 µm, polyethersulfone; Millipore, Carrigtwohill, Ireland). SE-HPLC was conducted using a LC-2010 system (Shimadzu, Kyoto, Japan) with automated injection monitoring at 214 nm. Protein extracts were loaded on a Biosep-SEC-S4000 column (pore size 500 Å, Phenomenex, Torrance, CA, USA). Injection volume was 60 µl unless specified otherwise.

**Elution media** (flow rate 1.0 ml/min, 30 °C) were (i) acetonitrile/water (1:1, v/v) containing 0.05% (v/v) trifluoroacetic acid [ACN medium, pH 2.25 after correction for measurements in acetonitrile/water mixtures (Gagliardi *et al.* 2007)], (ii) SDS medium and (iii) 0.100 M sodium phosphate buffer (pH 6.8) with 2.0% (w/v) SDS. The relative area was calculated from the peak area expressed as a percentage of the area of proteins extracted in SDS/urea/DTT medium. After analyses with SDS containing eluents, the column was cleaned overnight with water to avoid SDS contamination. No irreversible modification of the Biosep-SEC-S4000 column performance as a result of SDS use was noticed.

### 3.2.4 Protein extractability

Proteins (10 mg protein/ml extraction medium) from gluten, soy glycinin, BSA and ovalbumin samples were extracted/dissolved and centrifuged as in section 3.2.3. The protein contents of the supernatants of these extracts were then determined as in section 3.2.2. Protein extractability was defined as the percentage of the protein content of an extract relative to the total protein content.

### 3.2.5 Lab-on-a-Chip capillary electrophoresis

To 7.0 mg of BSA or ovalbumin, 1.0 ml SDS medium was added and samples were shaken (30 min, room temperature). After centrifugation (13 000 *g*, 10 min), 8 µl supernatant was mixed with 4 µl of Agilent sample buffer (Agilent Technologies, Santa Clara, CA, USA) and heated at 100 °C for 5 min. After cooling, 168 µl deionized water was added. The mixture (6 µl) and MW markers were applied on an Agilent LabChip of a protein 230 kit and analyzed with an Agilent 2100 Bioanalyzer system to obtain electrophoresis patterns.

### 3.2.6 Statistics

Linear regression was used to determine slopes, intercepts and standard errors of calibration curves. Slopes were compared with an ANCOVA method ($P < 0.05$) using JMP® Pro 11.2.0 (SAS institute, Cary, NC, USA).

## 3.3 Results and discussion

### 3.3.1 Impact of elution media on-size effects for proteins extracted/dissolved in sodium dodecyl sulfate medium

SDS medium yields high extraction/solubility levels of various protein types without breaking covalent bonds (Table 3.2). Here, the impact of elution media on non-size effects for proteins extracted/dissolved in SDS medium was investigated.

**Table 3.2: Extractability/solubility (%) of proteins in water, salt medium, sodium dodecyl sulfate (SDS) medium and SDS/dithiothreitol (DTT) medium. Values were determined with an automated Dumas protein analysis system. Standard deviations are between brackets.**

| Sample | Water | Salt medium | SDS medium | SDS/DTT medium |
|---|---|---|---|---|
| Bovine serum albumin | 97 (0) | 95 (4) | 101 (1) | 102 (2) |
| Hen egg ovalbumin | 103 (2) | 96 (3) | 103 (1) | 104 (4) |
| Wheat flour | 20 (1) | 13 (1) | 75 (1) | 103 (1) |
| Wheat gluten | 20 (1) | 14 (4) | 81 (5) | 100 (1) |
| Soy glycinin | 6 (1) | 87 (4) | 101 (2) | 101 (2) |

First, BSA, ovalbumin and gluten proteins were extracted with SDS medium (1 mg protein per ml) and chromatographically separated with ACN medium as elution medium, *i.e.* a typical eluent for both globular (Léonil *et al.* 2000) and cereal (Batey *et al.* 1991) proteins. Total UV absorbance was fitted as a function of injected extractable protein (Table 3.3). The formula by Kuipers and Gruppen (2007) shows that the absorbance at 214 nm per mass unit protein is almost equal for BSA, ovalbumin and gluten protein. Nevertheless, the slopes of the calibration curves were higher for BSA and gluten than for ovalbumin (Table 3.3; ANCOVA test with $\alpha = 0.05$). Ovalbumin was underestimated. In addition, the resolution of the SE profile was poor (Figure 3.1.A). Ovalbumin either interacted with the column or precipitated in ACN medium. To investigate the likelihood of these phenomena, the ovalbumin extract was injected (2 µl) over the bypass, *i.e.* not separated over the column, using either ACN or SDS medium as elution solvent. The total absorbance in the ACN medium was 96% of that in SDS medium. Thus, ovalbumin precipitation in the eluent was limited – if any. Instead, ovalbumin underestimation was mainly caused by interactions between the protein and the column. SE profiles of BSA showed three peaks (Figure 3.1.A) while Lab-on-a-Chip electrophoresis only revealed dimers and monomers (Figure 3.2.A). The partially postponed elution of BSA was a non-size effect (Figure 3.1.A). For gluten proteins, no non-size effects were noted. SDS-extractable glutenin eluted between 5 min and 6 min 40 sec, gliadin between 6 min 40 sec and 8 min 5 sec. Each protein has a unique pattern of surface charge distribution and hydrophobicity (Dubin *et al.* 1993). Dubin *et al.* (1993) demonstrated that not the net protein charge but the mean surface potential determines interactions of proteins with the resin.

**Table 3.3: Regression data of the calibration curves obtained by fitting the total absorbance of detected protein as a function of injected extractable protein. Bovine serum albumin (BSA), wheat gluten and hen egg ovalbumin were extracted with sodium dodecyl sulfate (SDS) medium and separated by SE-HPLC using either acetonitrile (ACN) or SDS medium as eluent. Standard deviations are between brackets.**

| Sample | Slope (mg extracted protein$^{-1}$) | Intercept | Standard error | Data points* | Linear until |
|---|---|---|---|---|---|
| **ACN medium** | | | | | |
| BSA | 10.29 (0.14) | 0.007 (0.005) | 0.9949 | 19 | 0.060 mg |
| Gluten | 10.18 (0.13) | -0.001 (0.006) | 0.9956 | 20 | 0.100 mg |
| Ovalbumin | 4.78 (0.07) | 0.005 (0.002) | 0.9957 | 15 | 0.060 mg |
| **SDS medium** | | | | | |
| BSA | 10.64 (0.06) | 0.003 (0.001) | 0.9995 | 12 | 0.030 mg |
| Gluten | 10.70 (0.13) | 0.007 (0.006) | 0.9957 | 21 | 0.100 mg |
| Ovalbumin | 10.32 (0.09) | 0.016 (0.004) | 0.9979 | 17 | 0.080 mg |

***Data points within the linear range and measured at least in triplicate**

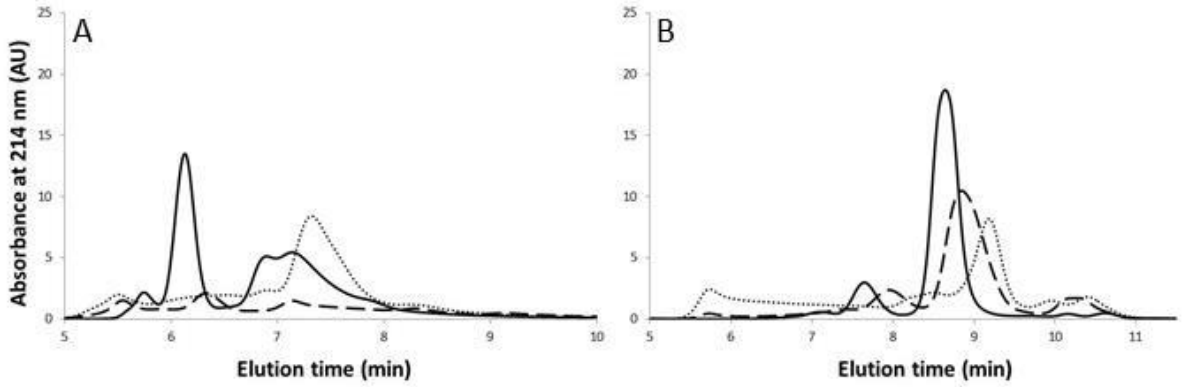


**Figure 3.1: SE-HPLC profiles of bovine serum albumin (—), hen egg ovalbumin (– –) and wheat gluten (····) extracted with sodium dodecyl sulfate (SDS) medium using acetonitrile (ACN) medium (A) or SDS medium (B) as eluent. AU, arbitrary units.**

Next, SDS medium was used both as extraction/solubilizing and elution medium. BSA mainly eluted as monomer at 8 min 40 sec, and partly as dimer at 7 min 40 sec (Figure 3.1.B) in agreement with Lab-on-a-Chip electrophoresis observations (Figure 3.2.A). Ovalbumin monomers and dimers, also observed by Lab-on-a-Chip electrophoresis (Figure 3.2.B), eluted at respectively 8 min 55 sec and 7 min 55 sec (Figure 3.1.B). Low levels of proteins eluted at 10 min 25 sec probably due to sample contamination (≥ 90% purity). SDS-extractable glutenin eluted between 5 min and 7 min 55 sec and gliadin between 7 min 55 sec and 9 min 36 sec (Figure 3.1.B). No indications for non-size effects were noted. Proteins eluted as expected based on their MW. However, a better resolution was obtained in SDS medium than in ACN medium. In contrast to what was the case with the first elution medium, the slopes of the calibration curves of BSA, ovalbumin and gluten were not significantly different (Table 3.3; ANCOVA method). However, the calibration curve of BSA was only linear up until 0.030 mg protein, while those of gluten and ovalbumin were linear up until 0.100 mg and 0.080 mg protein respectively. The slopes of the calibration curves of BSA with SDS medium as eluent were not

significantly different from those with ACN medium as eluent. In contrast, for ovalbumin and gluten the slopes with different media were significantly different (Table 3.3; ANCOVA method). The latter suggests even non-size effects for gluten in ACN medium.

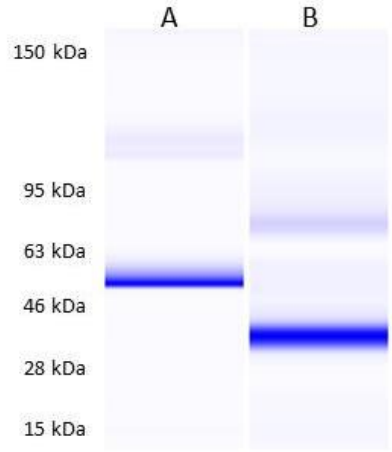


**Figure 3.2: Lab-on-a-Chip capillary electrophoresis pattern of bovine serum albumin (A) and hen egg ovalbumin (B) showing monomers and dimers of both proteins.**

Increasing the sodium phosphate concentration in the SDS medium from 0.050 M to 0.100 M decreased the absorbance per mass unit injected extractable protein BSA and gluten by respectively 49% and 59% (results not shown). Even after injection over the bypass, it already decreased the absorbance per mass unit injected extractable protein with *ca.* 33% and *ca.* 11% for BSA and gluten, respectively. Even though eluents usually have a salt concentration between 0.10 and 0.50 M (Stulík *et al.* 2003), our results show that such salt concentrations induced salting-out of proteins extracted in SDS medium. Salting out occurred as soon as the protein extracts were immersed in elution solvent, but even more during separation on column.

With ACN medium, all protein levels were (slightly) underestimated and BSA elution was postponed. No non-size effects were noted for any protein analyzed using SDS medium as extraction/solubilizing medium and eluent. The linear range depended on the protein. It was highest for gluten and lowest for BSA. Increasing the concentration of phosphate in the eluent caused salting out of all tested proteins.

### 3.3.2 Impact of extraction/solubilizing media on non-size effects with acetonitrile medium as eluent

This section investigated the impact of extraction media (Table 3.1) on non-size effects when ACN medium was used as eluent (Figures 3.3.A1 to 3.3.A5, Table 3.4) to increase the insight of co-solvents in extraction media and their working mechanisms on the separation of proteins. With this eluent, non-size effects were noted for proteins extracted in SDS medium (cfr. Section 3.3.1).

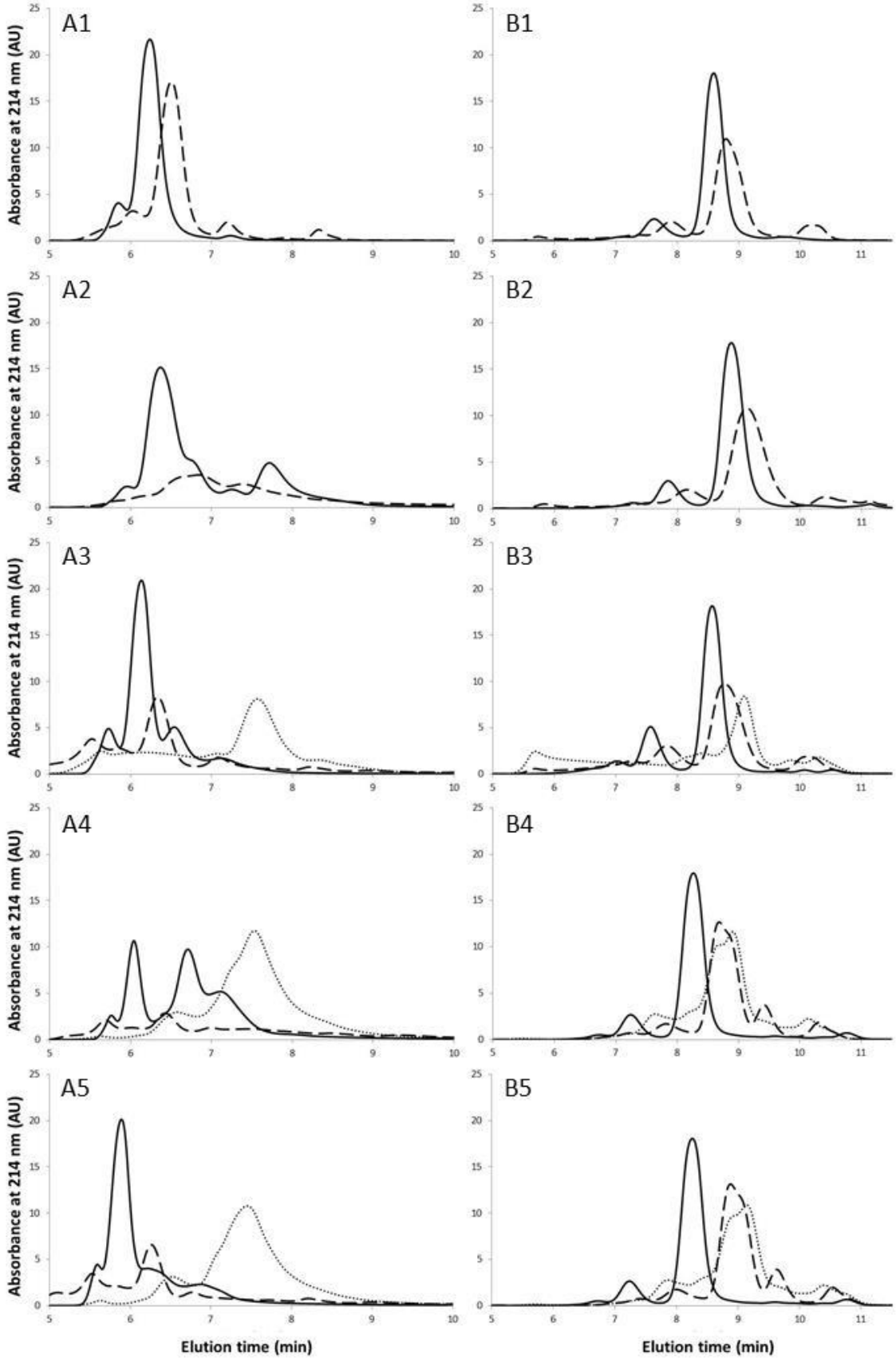


**Figure 3.3: SE-HPLC profiles of bovine serum albumin (—), hen egg ovalbumin (– –) and wheat gluten (····) for extracts with water (1), salt medium (2), sodium dodecyl sulfate (SDS)/urea medium (3), SDS/dithiothreitol (DTT) medium (4) and SDS/urea/DTT medium (5) using acetonitrile (ACN) medium (A) or SDS medium (B) as eluent. AU, arbitrary units.**

The peak shapes for BSA and ovalbumin extracted in water did not reveal non-size effects (Figure 3.3.A1). However, BSA (66 kDa) and ovalbumin (43 kDa) eluted at the same time as glutenin (> 80 kDa) extracted with SDS medium (Figure 3.1.A) which is earlier than expected based on their MW. At pH 2.25, silanol residues of the resin have a net positive charge (Irvine 1987). Thus, in aqueous media repulsive ionic interactions decrease the affinity of BSA (pI 4.7) and ovalbumin (pI 4.5) for the resin. From the gluten and wheat flour samples, only albumins were extractable in water (Tables 3.2 and 3.4). Profiles of albumins extracted from the gluten sample in water were not shown in Figure 3.3.

**Table 3.4: Areas of various proteins extracted with water, salt medium, sodium dodecyl sulfate (SDS) medium, SDS/urea medium, SDS/dithiothreitol (DTT) medium and SDS/urea/DTT medium using acetonitrile (ACN) medium as eluent. AU, arbitrary units. Standard deviations are between brackets.**

| Sample | Water | Salt medium | SDS medium | SDS/urea medium | SDS/DTT medium | SDS/urea/DTT medium |
|---|---|---|---|---|---|---|
| Bovine serum albumin | 52 (2) | 69 (2) | 60 (2) | 62 (2) | 59 (1) | 58 (1) |
| Hen egg ovalbumin | 51 (1) | 30 (1) | 23 (0) | 45 (1) | 28 (1) | 35 (3) |
| Wheat flour | 13 (0) | 8 (0) | 47 (1) | 52 (0) | 66 (0) | 66 (1) |
| Wheat gluten | 12 (0) | 5 (0) | 51 (4) | 58 (0) | 73 (1) | 69 (1) |
| Soy glycinin | 1 (0) | 40 (1) | 58 (0) | 60 (3) | 55 (1) | 56 (0) |

As salts lower ionic interactions, their inclusion in the extraction medium reduced the repulsion between positively charged proteins and the column. The salts shifted the monomer peak of BSA from 6.2 min in water to 6.4 min (Figure 3.3.A2). However, salts had a huge negative impact on separation and detection (Table 3.4) of BSA and ovalbumin. Part of BSA eluted later than the remainder. The ovalbumin level was underestimated (Tables 3.2 and 3.4). At low pH (here 2.25), salts can induce denaturation, *i.e.* unfolding of proteins with loss of secondary and tertiary structure (Fink *et al.* 1994), and thereby increase the affinity of proteins for resins. In addition, at low ionic strength salts can bind as counter ions and reduce repulsive interactions between resin and protein (Golovchenko *et al.* 1992). Thus, more non-size effects occurred in salt medium than in water, and they were caused both by ionic and hydrophobic interactions.

For globular proteins, *e.g.* BSA, ovalbumin and soy glycinin extracted in SDS medium, the same non-size effects occurred as in salt medium but more prominently (Figure 3.1.A). The anionic detergent SDS binds proteins reversibly through hydrophobic and ionic interactions (Otzen 2011). It strongly interacts with oppositely charged globular proteins (Jones and Manley 1980) like ovalbumin and BSA at pH 2.25. By giving protein a negative charge, it increases its affinity for the positively charged resin resulting in postponed elution and even reduced detection. However, within one protein the affinity of SDS differs for various sites (Otzen 2011). That only a fraction of BSA eluted later (Figure 3.1.A) can

be explained by the different degrees of denaturation of both secondary and tertiary structures induced by SDS binding to various protein sites. SDS increased the extractability of soy glycinin and gluten proteins by breaking non-covalent interactions (Table 3.2). While SDS was essential for extracting gluten proteins, it caused non-size effects for globular proteins by disrupting their structure.

The inclusion of urea in SDS medium decreased non-size effects for BSA and ovalbumin (Figures 3.3.A3 and 3.1.A). It minimized the shift of BSA to higher retention times and increased the total area of ovalbumin (Table 3.4). SDS and urea interact with different protein sites (Otzen 2011). While urea unfolds proteins, reduces the $\alpha$-helicity and completely disrupts $\beta$-structures (Bennion and Daggett 2003), the impact of SDS on the conformation of globular proteins depends on the presence of urea (Moriyama *et al.* 1993; Moriyama and Takeda 1999). In its absence, SDS reduces the $\alpha$-helicity of native BSA. In its presence, SDS can induce some non-native secondary structure (Moriyama *et al.* 1993; Moriyama and Takeda 1999). These differences in secondary structure probably alter the affinity for resins. Furthermore, urea may occupy protein binding sites which otherwise would be available for binding with resins. The use of urea increased the extractability of proteins from wheat flour and gluten (Table 3.4), more specifically that of glutenin (Figure 3.3.A3). No non-size effects for wheat flour and gluten were noted.

The inclusion of DTT in SDS medium increased the portion of BSA which eluted later (Figure 3.3.A4). In absence of DTT, SDS decreases the α-helical structure of BSA but conserves some secondary structure (Takeda and Moriyama 2007). Interactions of SDS with BSA are restricted as a result of its 17 SS bonds. When these are broken, BSA unfolds to a larger extent leading to sites which are better accessible for interactions with the column. For ovalbumin, SDS/DTT medium had no profound impact on peak distribution but slightly decreased the level of protein detected (Figure 3.3.A4). That ovalbumin has only one SS bond (Huntington and Stein 2001) may explain why DTT did not increase non-size effects for ovalbumin to the same extent as for BSA. With DTT, areas of extracts from wheat flour or gluten increased and more protein eluted later (Figure 3.3.A4). The reduction of SS bonds between GS increased the extractability of wheat flour and gluten proteins, as reflected in Table 3.4.

Under reducing conditions, inclusion of urea in SDS medium had the same impact as that under non-reducing conditions. It minimized the shift of BSA to higher retention times and increased the quantity of ovalbumin detected (Figure 3.3.A5, Table 3.4). The reduction of SS bonds in BSA and ovalbumin shifted their profiles to lower elution times due to their increased hydrodynamic volumes. Remarkably, DTT did not reduce all BSA and ovalbumin dimers. While SDS/DTT medium increased non-size effects for ovalbumin and BSA, SDS/urea/DTT medium had little impact on SE profiles. Urea

and SDS bind with different regions during protein unfolding (Otzen 2011), reducing the affinity for resins. Furthermore, some non-native secondary structure is created when both SDS and urea are present, even after reduction of SS bonds, thereby minimizing non-size effects. SDS/urea/DTT medium had the same impact on the SE profile of gluten as did SDS/DTT medium. It decreased the average MW and increased total detected area.

To conclude, with ACN medium as eluent proteins did not elute as expected based on their MW. Repulsive ionic interactions between the column and globular proteins extracted in water decreased their elution time. Salts in the extraction medium induced protein denaturation thereby improving the affinity for resins through both ionic and hydrophobic interactions. All globular proteins and most of the gluten proteins were extractable in SDS medium. However, non-size effects on globular proteins were even more pronounced in SDS than in salt medium due to the enhanced unfolding. Addition of urea to SDS medium reduced non-size effects on globular proteins. Urea probably occupies protein binding sites on the resin and induces some non-native secondary structure. DTT increased non-size effects on globular proteins. No non-size effects were noted for gluten proteins based on their SE profiles.

### 3.3.3 Impact of extraction/solubilizing media on non-size effects with sodium dodecyl sulfate medium as eluent

Figures 3.3.B1 to 3.3.B5 show the SE profiles of BSA, ovalbumin and gluten extracted in various media (Table 3.1) and separated with SDS medium as eluent. A better resolution was obtained for all proteins than with ACN medium as eluent (Figure 3.3).

Extracts of BSA and ovalbumin in water eluted as dimers and monomers (Figure 3.3.B1). Contamination of the commercial ovalbumin sample eluted at 10 min 20 sec. Addition of salts increased the elution times (Figure 3.3.B2). With SDS medium as eluent, ionized silanol residues repelled BSA and ovalbumin which also have a net negative charge at pH 6.8. Salts minimized ionic interactions. By shielding they increased the affinity of ovalbumin and BSA for the resin and hence the elution time. Nevertheless, salts in the extraction medium deteriorated neither separation nor detection.

With SDS medium as eluent, extracts of ovalbumin and BSA in SDS medium (Figure 3.1.B) resulted in SE profiles similar to those when using water as extraction medium. In SDS medium, both proteins and resin had a net negative charge. SDS/urea medium slightly increased the portion of oligomer fractions of ovalbumin and BSA but substantial differences were noted neither with regard to resolution, elution times, elution profile (Figure 3.3.B3) nor relative areas (Table 3.5).

**Table 3.5: Relative areas of various proteins extracted with water, salt medium, sodium dodecyl sulfate (SDS) medium, SDS/urea medium and SDS/dithiothreitol (DTT) medium using SDS medium as elution solvent. Injection volume was 20 µl. The relative area was calculated as a percentage of the corresponding peak area expressed on the area in SDS/urea/DTT medium. Standard deviations of triplicates are between brackets.**

| Sample | Water | Salt medium | SDS medium | SDS/urea medium | SDS/DTT medium |
|---|---|---|---|---|---|
| Bovine serum albumin | 96 (6) | 93 (2) | 96 (1) | 97 (5) | 98 (3) |
| Hen egg ovalbumin | 101 (4) | 90 (4) | 98 (8) | 101 (3) | 105 (8) |
| Wheat flour | 17 (0) | 15 (1) | 74 (2) | 78 (2) | 103 (5) |
| Wheat gluten | 15 (1) | 8 (1) | 82 (8) | 85 (5) | 102 (6) |
| Soy glycinin | 3 (0) | 70 (2) | 93 (6) | 96 (4) | 96 (4) |

Under reducing conditions, SE profiles of ovalbumin and BSA shifted to lower elution times (Figure 3.3.B4). The cleavage of intramolecular SS bonds made these proteins more accessible to denaturants thereby increasing their hydrodynamic volume. Remarkably, BSA and ovalbumin dimers were still present under reducing conditions. Probably, the compact structure of these proteins hindered the reduction of SS bonds. Inclusion of urea in SDS medium under reducing conditions impacted neither SE profiles (Figure 3.3.B5) nor relative areas of any protein (Table 3.5). This had been noted under non-reducing conditions as well.

With SDS medium as eluent, co-solvents in the extraction medium did not induce non-size effects.

## 3.4 Conclusion

This work demonstrated the importance of selecting appropriate eluents for analyzing proteins by SE-HPLC. While albumins and globulins are completely extractable in respectively water and aqueous salt solutions, gluten proteins require an extraction medium with co-solvents such as the denaturing agent SDS. Co-solvents in extraction media impact the separation of proteins, especially for albumins and globulins, with ACN medium as the eluent. Salts and SDS increase while urea decrease non-size effects. In contrast, with SDS medium as elution solvent, co-solvents in extraction media had substantial impact neither on peak shape and resolution, protein levels, nor on elution time.

Thus, heat-induced changes in MW distribution of different food proteins can be analyzed using SDS medium both as extraction and elution medium. Under the experimental conditions used, the ideal injecting volume was 20 µl of a protein solution containing 1.00 mg protein/ml. The chromatographic method optimized here will be successfully applied to extract and separate proteins in complex model systems (see Chapters 4 and 5) or from egg noodles (see Chapters 6 and 7).

# Chapter 4

# Thermodynamic compatibility of proteins: A case study in aqueous ethanol and water

## 4.1 Introduction

Interactions between amino acid side chains and their immediate environment affect protein aggregation. Small changes in temperature, pH, ionic strength and polarity can impact the conformation of globular proteins. While native and denatured proteins do not aggregate easily in aqueous environments due to buried hydrophobic regions, partially unfolded proteins with notable secondary structure are more prone to aggregate (Chi *et al.* 2003). Alcohols are less polar than water and thus weaken hydrophobic interactions and enhance polar interactions thereby facilitating protein denaturation (Thomas and Dill 1993). Often, proteins denature in aqueous-organic media but not in the corresponding pure organic solvent (Griebenow and Klibanov 1996). Furthermore, especially the larger alkyl alcohols stabilize α-helical conformations of unfolded proteins (Hirota *et al.* 1997). Because aqueous alcohols partially unfold proteins, they can induce protein aggregation (Singh *et al.* 2010). With increasing ethanol concentration, BSA tends to lose its secondary structure and form aggregates (Liu *et al.* 2010). Similarly, with increasing alcohol concentrations, partial and

This chapter is based on the following reference: Lambrecht M.A., Rombouts I., Delcour J.A. **2016**. Denaturation and covalent network formation of wheat gluten, globular proteins and mixtures thereof in aqueous ethanol and water. Food Hydrocolloids 57: 122-131.

progressive dehydration and alcohol binding transforms gel-like sediments of milk and soy proteins into opaque flocks (precipitates) (Boulet *et al.* 2001). It is clear neither whether alcohol-induced aggregation of albumins and globulins in the above examples is due to non-covalent interactions or covalent cross-links, nor whether and how alcohols would influence heat-induced aggregation.

While food systems often contain more than one protein type, protein denaturation and polymerization have mainly been studied in single protein systems. Due to differences in solubility, proteins in complex food systems can be present in various phases. In this context, Polyakov *et al.* (1997) used the term protein thermodynamic incompatibility. They even stated that differences in hydrophilic character between various protein types trigger phase separation and thereby promote interactions between proteins with similar conformation (Section 2.4). Given the impact of alcohols on protein conformation and solubility, it is of interest to compare heat-induced polymerization of complex systems in water to that in aqueous ethanol.

Against this background, the first objective of this Chapter was to compare structural changes, SH oxidation, SH-SS interchange and β-elimination reactions during heat treatment of various proteins in aqueous ethanol to those in water. BSA and glycinin were chosen as model globular proteins. Gliadin was selected as model gluten protein. The second objective was to evaluate the impact of different protein types on each other's denaturation and polymerization during heating in water and aqueous ethanol. Here, the importance of protein incompatibility during heat treatment of complex systems containing different protein types was investigated. It is hypothesized that two protein types in the same system influence each other's polymerization more when both are in solution than when they are phase-separated. The third objective was to study the impact of a pretreatment or isolation step with aqueous ethanol on the polymerization behavior of isolated proteins and mixtures thereof.

## 4.2 Materials and methods

### 4.2.1 Materials

**Gluten** (83.2% protein on dm) and **soy glycinin** (98.1% protein on dm) were isolated as in Section 3.2.1. Gliadin was extracted from gluten (20.0 g) with 70% (v/v) ethanol (250 ml). After centrifugation (10 000 *g*, 10 min), ethanol was evaporated (Rotavapor R3000, Büchi, Flawil, Switzerland) from the supernatant. **Gliadin** (87.7% dm protein) was freeze dried, ground in a laboratory mill (IKA), and passed through a 250 µm sieve. **BSA** (fraction V for biochemistry, 98.2% protein on dm) was from Acros Organics. Soy glycinin, BSA and gliadin were chosen as model proteins because they are colloidally stable in 50% (v/v) ethanol. All chemicals were at least of analytical grade and from Sigma-Aldrich unless specified otherwise. DTT, disodium hydrogen

phosphate and sodium dihydrogen phosphate were from VWR International. The protein contents were determined in triplicate as described in Section 3.2.2.

### 4.2.2 Aqueous ethanol pretreatment

BSA, soy glycinin and gluten (500.0 mg dm protein) were shaken in triplicate for 60 min with 5.0 ml 50% (v/v) ethanol. Gluten (500.0 mg dm protein) was also pretreated with 70% (v/v) ethanol (5.0 ml) in a similar way to simulate conditions during gliadin isolation. Ethanol was evaporated from samples using a Rotational Vacuum Concentrator (Q-lab, Vilvoorde, Belgium, 35 °C, 1.0 mbar). Aqueous ethanol pretreated (EtPT) samples were freeze-dried and ground using a mortar and pestle.

### 4.2.3 Heat treatment

Deionized water, 10% or 50% (v/v) ethanol (5.0 ml) were added to BSA, soy glycinin or gliadin (500.0 mg or 166.7 mg dm protein). Furthermore, blends of either gluten or gliadin with BSA or soy glycinin [500.0 mg or 166.7 mg protein in total, ratio (2:1)] were mixed with 5.0 ml deionized water or 50% (v/v) ethanol. Isolated proteins, including EtPT proteins and mixtures thereof were suspended in water. The reaction tubes (glass, inner diameter = 27 mm, outer diameter = 34 mm, height = 100 mm) were hermetically sealed and horizontally shaken at 100 °C for 6, 60 or 120 min at least in duplicate. Heat-treated samples were immediately cooled in water. The heating temperature (100 °C) was chosen because of its relevance for pasta and noodle products. Furthermore, under the conditions used the globular proteins are denatured at this temperature. Unheated samples were shaken for 60 min at room temperature. Samples were considered colloidally stable if their proteins in a stirred reaction mixture did not precipitate after three days of standing at room temperature. Ethanol was evaporated from samples using a Rotational Vacuum Concentrator (35 °C, 1.0 mbar). All samples were freeze-dried and ground using a mortar and pestle.

### 4.2.4 Size exclusion high performance liquid chromatography

SE-HPLC was conducted as optimized in Chapter 3. This method was repeatable when applied to samples heated on different days. To extract proteins under non-reducing conditions, samples (1.0 mg protein) were shaken (60 min, room temperature) with 1.0 ml SDS medium. Proteins were also extracted under reducing conditions, *i.e.* under nitrogen atmosphere using SDS/DTT medium. All extractions were in triplicate. After centrifugation (10 000 *g*, 10 min) and filtration (0.45 µm) extracts (20 µl) were loaded on a Biosep-SEC-S4000 column. The eluent was SDS medium (flow rate 1.0 ml/min, 30 °C). Protein elution was monitored at 214 nm. The level of protein extractable in SDS medium (SDS-EP) under non-reducing conditions was calculated from the corresponding peak area and expressed as a percentage of the assumed total area. The assumed total area of a sample was

that of the corresponding unheated sample extracted under reducing conditions. SDS-extractable polymeric compounds were collected, reduced with DTT (room temperature, 60 min) and again separated using SE-HPLC.

### 4.2.5 Differential scanning calorimetry

Protein denaturation properties were determined at least in triplicate with a Q2000 DSC (TA instruments, New Castle, DE, USA). Samples were accurately weighed (2.20-4.00 mg) in an aluminum pan (Perkin-Elmer, Waltham, MA, USA) and high pressure steel pans (Mettler-Toledo, Zaventem, Belgium) when analyzed in deionized water on the one hand and in aqueous ethanol [10% or 50% (v/v) ethanol] on the other. Pans [1/3 (w/w) protein/solvent] were hermetically sealed and heated from 0 °C to 120 °C at 4 °C/min. Empty pans were used as reference. Calibration was with indium. The denaturation onset, peak, conclusion temperatures, temperature ranges and enthalpies were determined using Universal Analysis 2000 software (TA Instruments).

### 4.2.6 Determination of free sulfhydryl content

Free SH groups were determined colorimetrically with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Samples (1.0-1.5 mg protein) were shaken (10 min, room temperature) with 200 µl water or 50% (v/v) ethanol. Afterwards, 800 µl sample buffer [0.050 M sodium phosphate buffer (pH 6.5) containing 2.0% (w/v) SDS, 3.0 M urea and 1.0 mM tetrasodium ethylenediaminetetraacetate] and 100 µl DTNB reagent [0.1% (w/v) in sample buffer] were added and the samples were shaken. The absorbance at 412 nm was read exactly 10 min after adding DTNB reagent. Absorbance values were converted to concentrations of free SH using a calibration curve with reduced glutathione. Controls without DTNB or sample were used to correct for background absorbance of DTNB and sample. All analyses were performed in triplicate.

### 4.2.7 Determination of oxidation reaction rate

DTT (0.05 mg/ml) was dissolved in water, 10% or 50% (v/v) ethanol and heated for several hours at 100 °C. After cooling on ice (5 min), sample (200 µl) was added to 800 µl sample buffer (as described in section 4.2.6) and 100 µl DTNB [0.1% (w/v) in sample buffer] reagent. After exactly 10 min, the absorbance at 412 nm was read. All analyses were performed in triplicate.

### 4.2.8 Determination of sulfhydryl-disulfide exchange reaction rate

Rates of SH (DTT)-SS (DTNB) exchange reactions were monitored in triplicate under nitrogen atmosphere. To 900 µl DTT [0.25% (w/v) DTT in 1.0% (w/v) SDS with 0.83 M urea], 100 µl DTNB [0.1% (w/v) in 1.0% SDS (w/v) with 0.83 M urea] and 1000 µl solvent [1.0% (w/v) SDS in water and/or 1.0% SDS (w/v) in ethanol] were added to a final concentration of 0%, 10% and 50% (v/v) ethanol.

The absorbance readings at 412 nm were monitored for 10 min at room temperature. The absorbance of the first measurements was used to correct for background absorbance. Results were corrected for the difference in extinction coefficient of DTNB in aqueous ethanol compared with water.

### 4.2.9 Analysis of lysinoalanine and lanthionine cross-links

The DHA-derived cross-links LAN and LAL were quantified in gliadin after heating (100.0 mg dm protein/ml solvent, in triplicate) in water or 50% (v/v) ethanol for 15 h at 130 °C. After ethanol evaporation (Rotational Vacuum Concentrator, 35 °C, 1.0 mbar), samples were freeze-dried and ground. (Iso)peptide bonds were hydrolyzed by heating at 110 °C for 24 h in 6.0 M HCl and (cross-linked) amino acids were separated and quantified using high-performance anion-exchange chromatography with pulsed amperometric detection as in Rombouts *et al.* (2009).

### 4.2.10 Lab-on-a-Chip capillary electrophoresis

Soy glycinin in 50% (v/v) ethanol (20.0 mg protein/ml) was heated for 5 min at 70 °C. After centrifugation (10 000 *g*, 10 min) supernatant and precipitate were separated and freeze-dried. To these samples and isolated glycinin (7.0 mg protein), 1.0 ml sodium phosphate buffer (0.050 M; pH 6.8) containing 2.0% (w/v) SDS was added, samples were shaken (30 min, room temperature) and analyzed as described in Section 3.2.5.

### 4.2.11 Circular dichroism

Circular dichroism (CD) spectra of (EtPT) BSA in water and 50% (v/v) ethanol were recorded in the far-UV range (190 to 250 nm) at room temperature with a Jasco J-810 Spectropolarimeter (Jasco Benelux, Maarssen, The Netherlands) using a quartz cell with 1.0 mm path length and a protein concentration of 0.1 mg/ml. Data were expressed as molar residual ellipticity [$\Theta$], defined as

$$\theta = \frac{100\,[\theta]_{obs}}{\mathrm{l\,c}} \qquad \text{(Equation 4.1)}$$

were $[\Theta]_{obs}$ is the observed molar ellipticity in degrees, l the length of the cell light path in centimeters and c the protein concentration in mol/l.

### 4.2.12 Statistics

Significant differences ($\alpha = 0.05$), based on at least three measurements, were determined with the one-way ANOVA procedure using JMP® Pro 11.2.0 (SAS Institute). Corresponding Tukey grouping coefficients are given.

## 4.3 Results and discussion

### 4.3.1 Impact of water and aqueous ethanol on protein network formation

After shaking for 60 min at room temperature in water or 10% (v/v) ethanol, freeze-drying and subsequently extracting with SDS medium, both monomeric and dimeric BSA were found (Figure 4.1.1). In comparison, BSA in 50% (v/v) ethanol yielded, beside monomers and dimers, both extractable and non-extractable higher MW compounds. Water and 10% (v/v) ethanol had no impact on the SDS-EP content of unheated BSA (100% ± 7%) while 50% (v/v) ethanol reduced it to 68% ± 6%. Under reducing conditions, all BSA samples were fully extractable in SDS medium and only consisted of monomers and dimers (results not shown). The above demonstrate that SS cross-links were formed between BSA molecules at 50% (v/v) ethanol already at room temperature. Liu *et al.* (2010) reported the formation of larger (100 nm) BSA aggregates in 50% (v/v) ethanol. Our results demonstrate that not only non-covalent interactions but also covalent cross-links contribute to such aggregates. Liu *et al.* (2010) reported that the helicity of BSA decreased when the ethanol concentration increased from 10% (v/v) until complete unfolding was observed at 50% (v/v) ethanol. In agreement, DSC analyses of BSA (Figure 4.2.A) showed that the denaturation temperatures and enthalpies decreased with increasing ethanol concentration until complete denaturation at 50% (v/v) ethanol. It is reasonable to assume that the unfolding induced by aqueous ethanol facilitates SS cross-linking. At room temperature, SH-SS exchange reactions occurred faster with decreasing ethanol concentration (Figure 4.3). In addition, more free SH groups of BSA were accessible in 10% (5.1 ± 0.9 µmol SH/g protein) and 50% (v/v) ethanol (4.5 ± 0.4 µmol SH/g protein) than in water (2.5 ± 0.3 µmol SH/g protein). Furthermore, to polymerize, proteins have to overcome an energy barrier mainly formed by electrostatic and van der Waals interactions. The immediate environment of protein impacts its colloidal stability and thus its tendency to precipitate (Chi *et al.* 2003). At room temperature, BSA (100.0 mg protein/ml) was colloidally stable in water and 10% (v/v) ethanol but not in 50% (v/v) ethanol in which it also polymerized. Heating BSA (100.0 mg protein/ml) in either water, 10% or 50% (v/v) ethanol rapidly reduced its SDS-EP content to 3% ± 1% (Figure 4.1.1). At a lower concentration (33.3 mg protein/ml), BSA was colloidally stable and fully extractable in SDS medium at room temperature in both water and 50% (v/v) ethanol. However, more polymers extractable in SDS medium were formed in 50% (v/v) ethanol than in water (Figure 4.4).

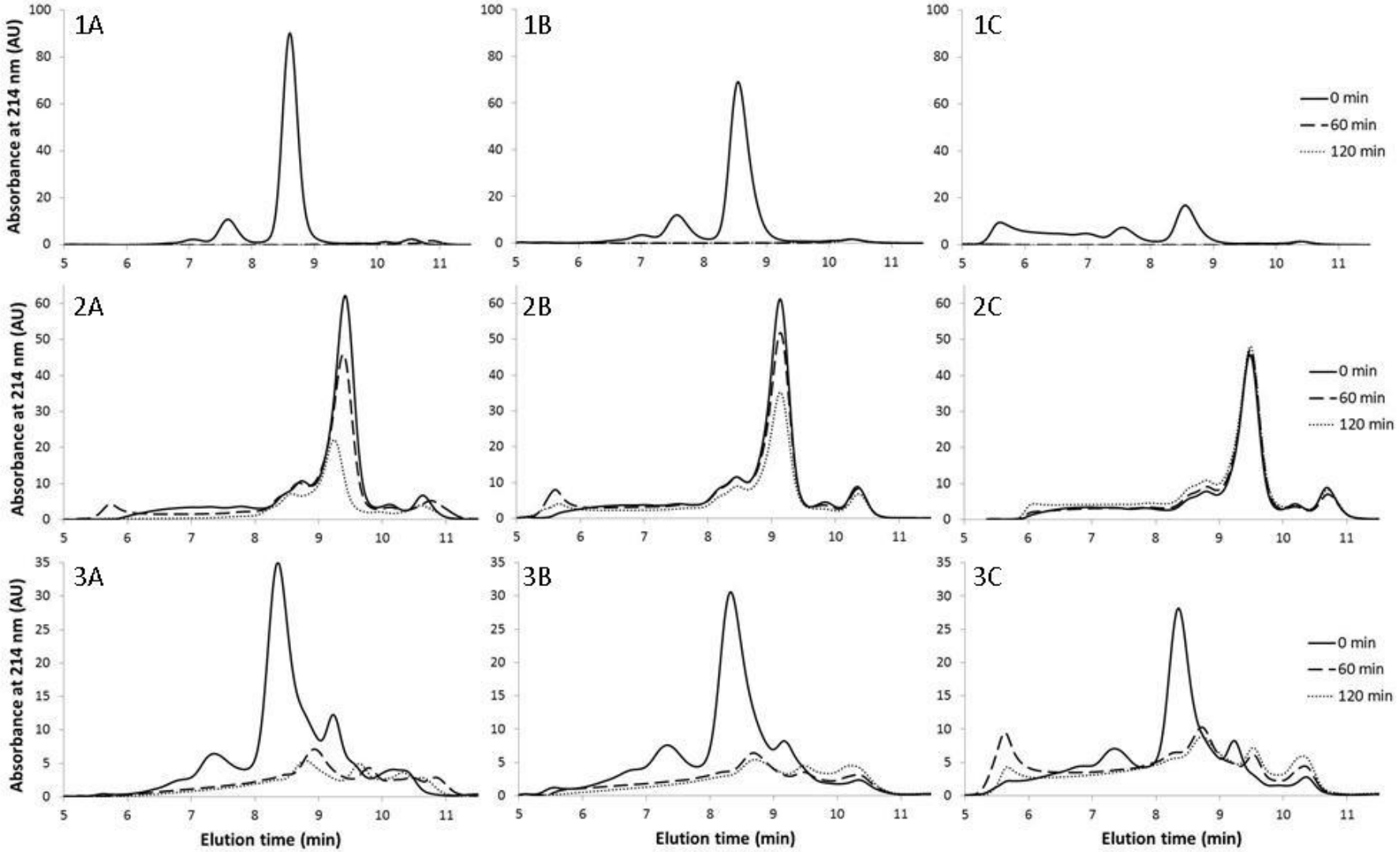


**Figure 4.1: SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of bovine serum albumin (BSA, 1), gliadin (2) and soy glycinin (3) before and after heat treatment at 100 °C for 60 and 120 min in water (A), 10% (v/v) ethanol (B) and 50% (v/v) ethanol (C). During heating a concentration of 100.0 mg protein/ml solvent A, B or C was used. AU, arbitrary units.**

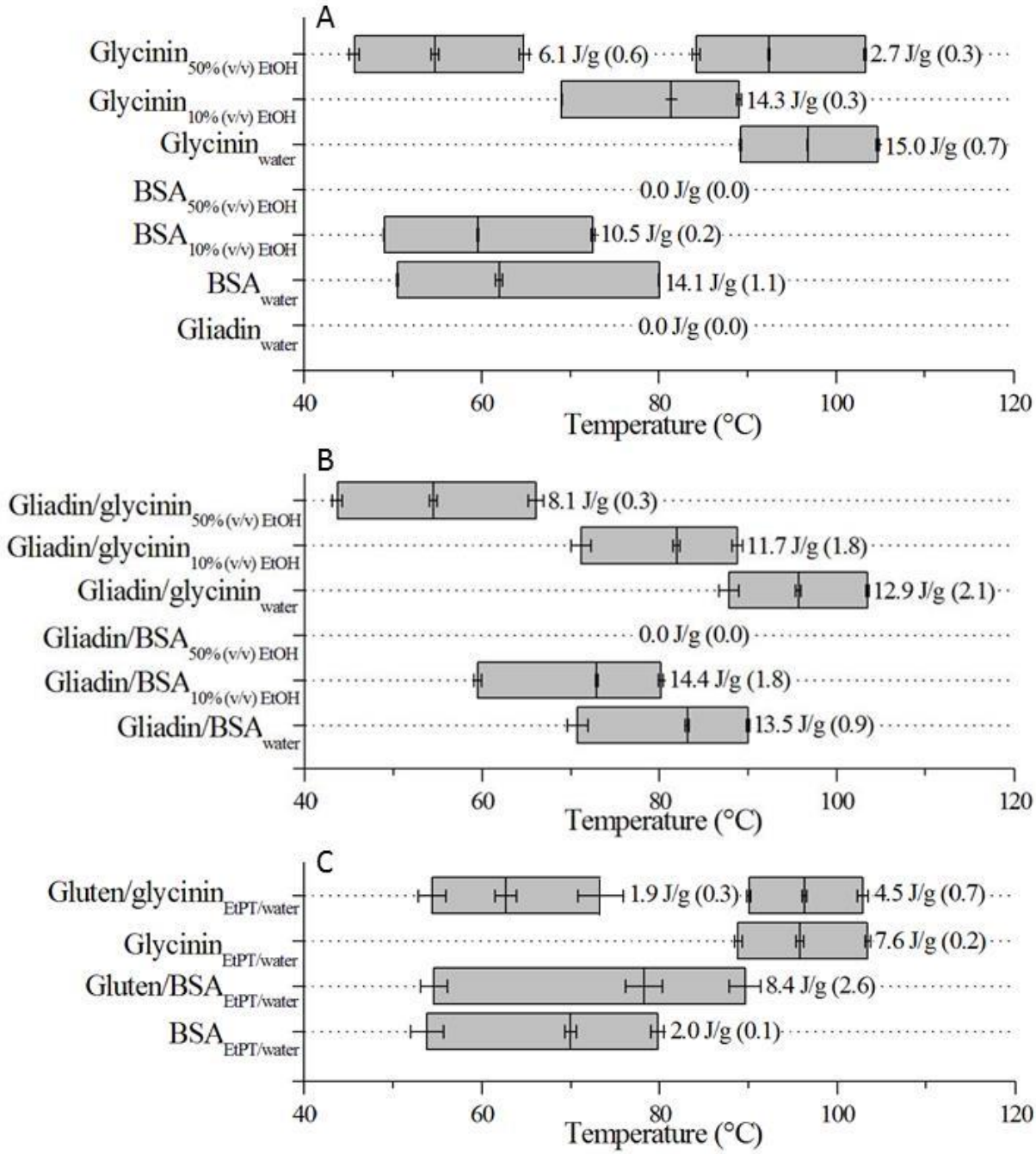


**Figure 4.2: Schematic overview of the denaturation temperature ranges and enthalpic transition data of bovine serum albumin (BSA), soy glycinin (A) and mixtures thereof with gliadin or wheat gluten (ratio 1:2, B) in water, 50% and 10% (v/v) ethanol (EtOH) and in water after pretreatment with 50% (v/v) ethanol (EtPT, C). Start and end points of the bars represent the denaturation onset and conclusion temperatures. The vertical lines inside the bars are the denaturation peak temperatures. The enthalpy data are expressed on the amount of globular proteins in the sample. Standard deviations are given between brackets.**

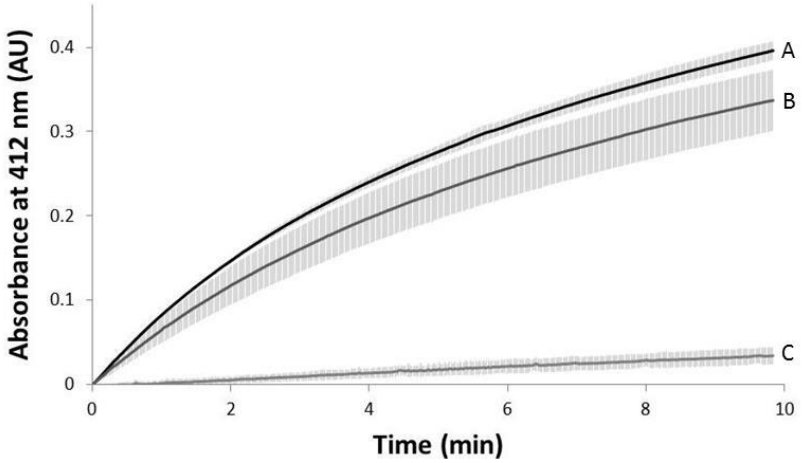


**Figure 4.3: Absorbance measurement in time of the sulfhydryl (SH)-disulfide (SS) exchange reaction between dithiothreitol (DTT) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in water (A), 10% (v/v) ethanol (B) and 50% (v/v) ethanol (C). AU, arbitrary units. Standard deviations are given as error bars.**

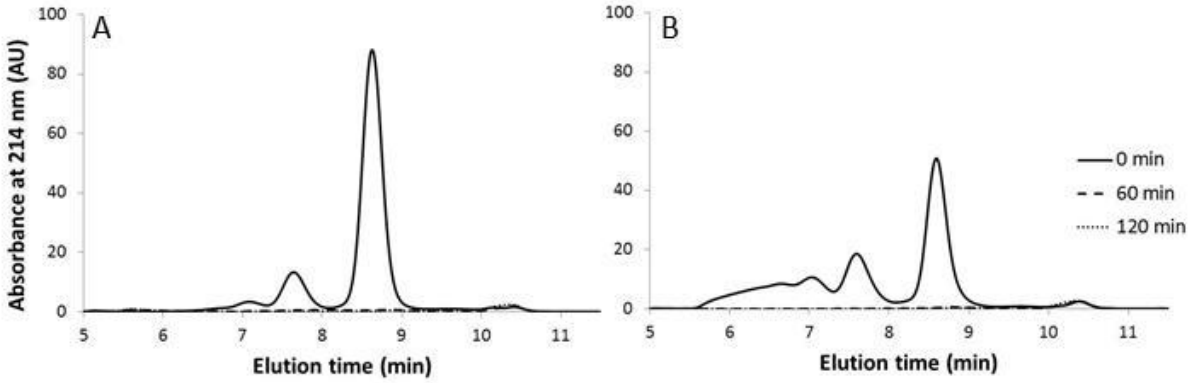


**Figure 4.4: SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of bovine serum albumin (BSA) heated in water (A) and 50% (v/v) ethanol (B) at a concentration of 33.3 mg protein/ml solvent. AU, arbitrary units.**

Gliadin, shaken for 60 min at room temperature in water or 10% (v/v) ethanol and subsequently extracted in SDS medium, eluted over a wide range in SE-HPLC. The largest gliadins (ω-gliadins) eluted between 8 min 20 sec and 8 min 55 sec, while most α- and γ- gliadins eluted between 8 min 42 sec and 9 min and 36 sec. While 10% (v/v) ethanol had significant impact neither on the MW nor on the extractability of gliadin in SDS medium at room temperature (Figure 4.1.2), it substantially reduced heat-induced polymerization compared to water as solvent. In 50% (v/v) ethanol, unheated gliadin was colloidally stable and the extractability of gliadin even remained constant during heating at 100 °C (Figure 4.1.2). SH oxidation occurred faster with decreasing ethanol concentration during heating at 100 °C (Figure 4.5). Gliadin lacks free SH groups. Its polymerization in water is initiated by β-elimination reactions from intramolecular SS bonds in α- and γ-gliadins which form DHA and free SH groups (Rombouts *et al.* 2010). The lack of polymerization of gliadin in 50% (v/v) ethanol indicates

that the extent of β-elimination reactions is limited. However, LAN, the product from Michael addition of the SH group from cysteine to DHA, was detected after severe and prolonged heating [15 h, in water or 50% (v/v) ethanol at 130 °C] of gliadin. This indicates that severe heating in 50% (v/v) ethanol was still able to induce β-elimination reactions. ω-Gliadins, which lack SS bonds, remained extractable during heating in SDS medium in either solvent. The slight heat-induced reduction of this peak is due to the polymerization of some α- and γ-gliadins co-eluting with ω-gliadins.

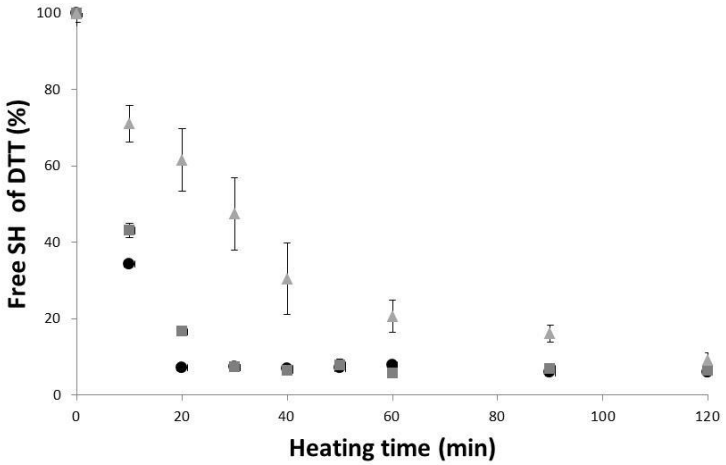


**Figure 4.5: Decrease in free sulfhydryl (SH) content of dithiothreitol (DTT) heated at 100 °C in water (○), 10% (v/v) ethanol (□) or 50% (v/v) ethanol (Δ). Standard deviations are given as error bars.**

With increasing ethanol concentration, the level of soy glycinin polymers extractable in SDS medium (before 7 min 48 sec) at room temperature increased (Figure 4.1.3). As observed for BSA, the increased polymerization of soy glycinin was ascribed to conformational changes in 50% (v/v) ethanol. Glycinin contains *ca.* 20 SS bonds which are mostly buried in the interior of the protein, and no free SH groups (Draper and Catsimpoolas 1978). Sodium bisulfite reduced some of these SS bonds into free SH groups during the isolation protocol. However, no significant differences between the levels of accessible SH groups in water (1.7 ± 0.3 µmol cysteine/g protein) and in 50% (v/v) ethanol (1.8 ± 0.2 µmol cysteine/g protein) were noted. With increasing ethanol concentrations, the extractability loss during prolonged (> 6 min) heating decreased (Figure 4.1.3). Heating glycinin at 100 °C in 50%, 10% (v/v) ethanol or water reduced the SDS-EP content to 73% (± 2%), 43% (± 3%) or 37% (± 2%) after 60 min and to 65% (± 5%), 40% (± 2%) or 33% (± 1%) after 120 min, respectively. Also, SH oxidation, SH-SS exchange reactions and β-elimination reactions occurred slower with increasing ethanol concentration (Figures 4.3, 4.5 and 4.1.2). Furthermore, soy glycinin proteins precipitated in water and 10% (v/v) ethanol while they were stable in 50% (v/v) ethanol. Ethanol impacted not only the covalent cross-linking, but also the denaturation of soy glycinin. Glycinin denatured in water with a peak temperature of 96.8 °C ± 0.1 °C and in 10% (v/v) ethanol with a peak

temperature of 81.4 °C ± 0.1 °C (Figure 4.2.A). In 50% (v/v) ethanol, glycinin showed two endothermic DSC peaks and the total denaturation enthalpy was lower than that in water or 10% (v/v) ethanol (Figure 4.2.A). It was investigated whether those two peaks correspond to basic (*ca.* 20 kDa) and acidic (*ca.* 38 kDa) polypeptides. In water, glycinin consists of subunits, each containing a basic and an acidic polypeptide connected by an SS bond (except for the acidic polypeptide $A_4$) (Staswick *et al.* 1984) which is cleaved upon heating (Hashizume and Watanabe 1979). However, heating glycinin to 70 °C in 50% (v/v) ethanol produced supernatants and precipitates which both contained acidic and basic polypeptides, but also glycinin subunits (results not shown). As discussed for gliadin, β-elimination reactions only occur to a limited extent in 50% (v/v) ethanol. So, it is very unlikely that the two distinct denaturation peaks are due to cleavage of the SS bonds between acidic and basic polypeptides. Instead, it is hypothesized that in 50% (v/v) ethanol both hexameric and trimeric glycinin complexes exist. The latter denature at a lower temperature than the former (Lakemond *et al.* 2000a). No trimeric nor hexameric complexes were present in the unheated glycinin extract due to the presence of SDS. Soy glycinin monomers eluted between 7 min 48 sec and 9 min (Figure 4.1.3). Acidic and basic polypeptides were detected after 9 min. Protein compounds eluting before 7 min 48 sec were attributed to polymerization during shaking in the selected solvent.

### 4.3.2 Network formation between different proteins in water and aqueous ethanol

In water, unheated BSA was soluble while unheated gliadin precipitated. The SDS-EP values of all BSA-gliadin mixtures (ratio 1:2) heated in water at 100 °C were lower than expected based on the extractability losses of the isolated proteins (Figure 4.6.1A). Thus, BSA and gliadin impacted each other's polymerization. More heat-induced polymerization than expected based on the isolated proteins is here referred to as a positive co-protein effect. SE-HPLC showed that after 6 min of heating, a small peak containing SDS-extractable polymers eluted between 5 and 6 min which did not appear in the profiles of the (heated) isolated proteins (results not shown). These polymeric compounds consisted of both BSA and gliadin. That, in contrast, 6 min heating of isolated BSA already resulted in complete extractability loss, allowed concluding that gliadin slows down BSA polymerization. In support, addition of gliadin increased the denaturation temperature of BSA in water from 62.0 °C ± 0.4 °C (Figure 4.2.A) to 83.1 °C ± 0.2 °C (Figure 4.2.B). Rombouts *et al.* (2012c) already described that, under the conditions they used, gluten increases the denaturation temperature of BSA at pH 8.0 to *ca.* 83.7 °C. Apparently, gliadin can also stabilize BSA. As expected, after 60 min of heating, BSA was mostly incorporated in the protein network. Here, the difference between measured and expected SDS-EP levels was due to substantial gliadin polymerization (Figure

4.6.1A, peak II). BSA facilitated the incorporation of gliadin in the protein network through SH-SS interchange reactions. A small peak of protein eluting at *ca.* 8 min 40 sec remained after 120 min of heating, and corresponds to ω-gliadin.

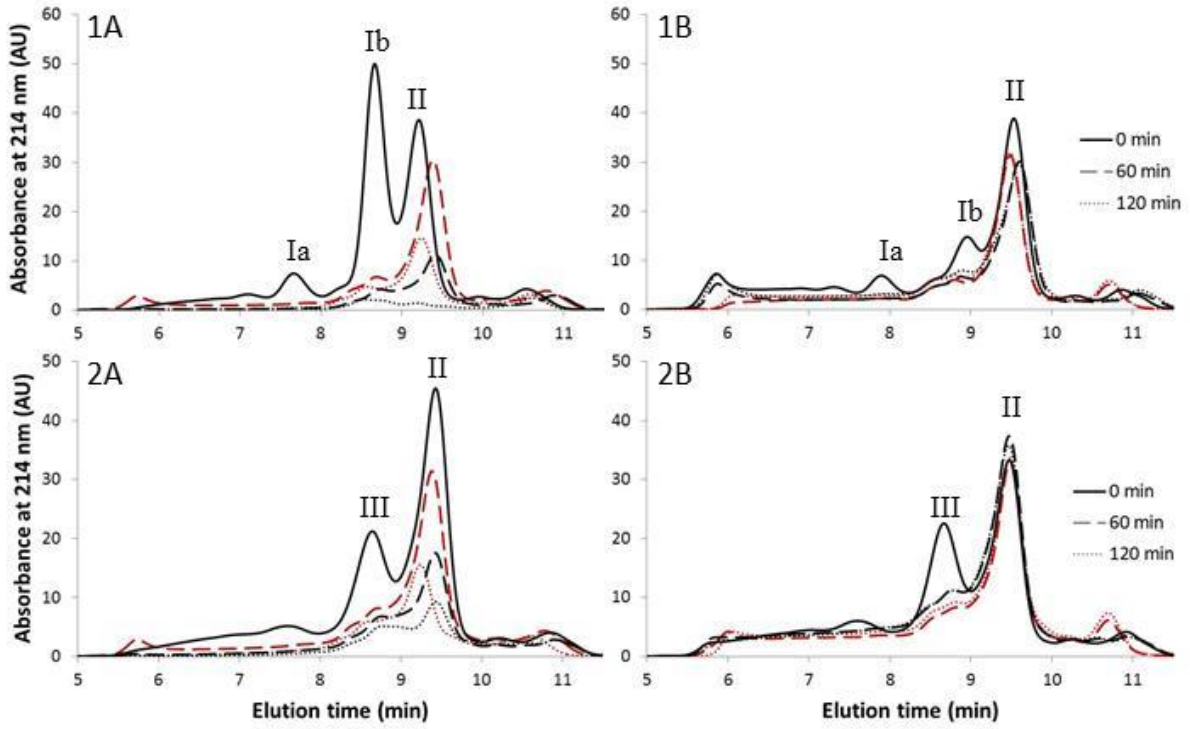


**Figure 4.6: SE-HPLC profile of protein extracts in sodium dodecyl sulfate medium (SDS-EP) of mixtures (ratio 2:1) of gliadin and bovine serum albumin (BSA, 1) and gliadin and soy glycinin (2) heated at 100 °C for different times in water (A) and 50% (v/v) ethanol (B). Peaks Ia and Ib are attributed to BSA dimers and monomers respectively. The main peaks of gliadin (II) and soy glycinin (III) are shown. Results for samples withdrawn after 60 (dashed, black) and 120 (dotted, black) min of heating are compared with calculated profiles based on data of the isolated proteins also after 60 (dashed, red) and 120 (dotted, red) min of heating. AU, arbitrary units.**

In 50% (v/v) ethanol, both BSA and gliadin were colloidally stable at room temperature. The total SDS-EP values of BSA-gliadin mixtures during prolonged (> 6 min) heating in 50% (v/v) ethanol were as expected based on the SDS-EP values of the isolated proteins (Figure 4.6.1B). At room temperature, BSA consisted of monomers, dimers and a wide range of (SDS-extractable) polymers in 50% (v/v) ethanol (Figure 4.1.1C). After 60 min of heating, little if any BSA monomer remained (peak overlap at *ca.* 8 min 40 sec with ω-gliadin) and gliadin ceased polymerizing (Figure 4.6.1B). The SDS-EP content of the BSA-gliadin mixture reached a plateau at *ca.* 66%.

In water, both soy glycinin and gliadin precipitated at room temperature. After heating in water, less protein was extracted in SDS medium from a glycinin-gliadin mixture than from the isolated proteins (Figure 4.6.2A). A positive co-protein effect occurred. Especially gliadin polymerized faster and to a larger extent with glycinin than alone. Addition of gliadin to glycinin (weight ratio 2:1) slightly

decreased the denaturation temperature of glycinin in water, but it did not change the associated enthalpy (Figure 4.2.B). Thus, when heated in water, glycinin impacted gliadin more than vice versa.

In 50% (v/v) ethanol, both unheated soy glycinin and gliadin were colloidally stable. After heating in 50% (v/v) ethanol, the glycinin-gliadin mixture had higher extractability than expected based on the extractability losses of the isolated proteins (Figure 4.6.2B). Either with or without glycinin, gliadin (Figure 4.6.2B, Peak II) remained fully extractable during heating in 50% (v/v) ethanol. In contrast, the extractability of glycinin (Figure 4.6.2B, Peak III) decreased less in the presence than in the absence of gliadin, in line with the impact of gliadin on the denaturation of glycinin in 50% (v/v) ethanol. In absence of gliadin, trimeric and hexameric glycinin complexes denatured at 54.5 °C and 92.5 °C, respectively, as stated in section 4.3.1 (Figure 4.2.A). In the presence of gliadin, all glycinin denatured at 54.5 °C, probably as trimeric complexes (Figure 4.2.B). It is hypothesized that the hexameric complexes, present in water with and without gliadin and to a small extent in 50% (v/v) ethanol without gliadin, but absent in 50% (v/v) ethanol with gliadin, contribute significantly to protein network formation. Thus, when heated in 50% (v/v) ethanol gliadin impacted glycinin more than vice versa.

Polyakov *et al.* (1997) described protein solubility as a key factor determining protein compatibility. Applying their theory to the present case, protein interactions between BSA and gliadin would be favored in 50% (v/v) ethanol, where both protein types are soluble, but not in water, where BSA and gliadin phase-separate. However, this work showed that both protein mixtures polymerize to a larger extent in water than in 50% (v/v) ethanol. The limited β-elimination, decreased SH oxidation and SH-SS exchange reaction rate, conformational changes in 50% (v/v) ethanol and the colloidal stability of proteins in the same medium, reduced covalent cross-linking. Moreover, in water, the proteins in both mixtures polymerized to a larger extent than expected based on the extractability losses of the isolated proteins, while in 50% (v/v) ethanol they polymerized equally or less than expected. Thus, a positive co-protein effect occurred in water and not in 50% (v/v) ethanol where both protein types are soluble.

### 4.3.3 Impact of aqueous ethanol pretreatment on protein network formation

We here evaluated the impact of the use of aqueous ethanol to isolate proteins, on their subsequent network formation and denaturation in water. At room temperature, EtPT did not affect the SDS-EP content of BSA in water (Table 4.1). Thus, while SS cross-links reduced the SDS-EP of BSA in 50% (v/v) ethanol to 68% (±6%) at room temperature (Section 4.3.1), subsequent drying and suspension of the sample in water restored the SDS-EP content, due to SH-SS exchange reactions which released BSA monomers from the protein network. Much as in 50% (v/v) ethanol, polymers, dimers and

monomers extractable in SDS medium were present in the unheated sample (profile not shown). However, EtPT slightly slowed down the extractability loss during heating, demonstrated by a higher SDS-EP content of BSA with than without EtPT after 6 min of heating (Table 4.1). Similar extents of polymerization were obtained with or without EtPT upon prolonged heating at 100 °C. EtPT also affected BSA denaturation (Figure 4.2.C). After denaturation in 50% (v/v) ethanol, suspension in water partly reversed denaturation. It yielded BSA which denatured at higher temperatures but with lower enthalpy. CD analyses also showed clear differences between the conformation of BSA in water and in 50% (v/v) ethanol, while that after EtPT was intermediate between both (Figure 4.7).

**Table 4.1: Proteins extractable in sodium dodecyl sulfate medium (SDS-EP, %, with standard deviations between brackets) of bovine serum albumin (BSA), soy glycinin and wheat gluten heated in water at 100 °C for various times with or without pretreatment with 50% (v/v) ethanol (EtPT).**

| Proteins | Heated in water | | | | EtPT and heated in water | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 min | 6 min | 60 min | 120 min | 0 min | 6 min | 60 min | 120 min |
| BSA | 94 (3)$^{a}$ | 5 (0)$^{b}$ | 3 (0)$^{a}$ | 3 (0)$^{b}$ | 84 (9)$^{a}$ | 8 (1)$^{a}$ | 3 (0)$^{a}$ | 4 (0)$^{a}$ |
| Soy glycinin | 97 (4)$^{a}$ | 92 (3)$^{b}$ | 37 (2)$^{a}$ | 33 (1)$^{a}$ | 96 (3)$^{a}$ | 97 (3)$^{a}$ | 34 (2)$^{a}$ | 35 (3)$^{a}$ |
| Wheat gluten | 75 (2)$^{a}$ | 52 (2)$^{a}$ | 31 (1)$^{a}$ | 25 (0)$^{a}$ | 73 (5)$^{a}$ | 45 (4)$^{b}$ | 30 (2)$^{a}$ | 24 (2)$^{a}$ |

**Results of the same protein and heating time indicated with the same letter are not significantly different (α = 0.05).**

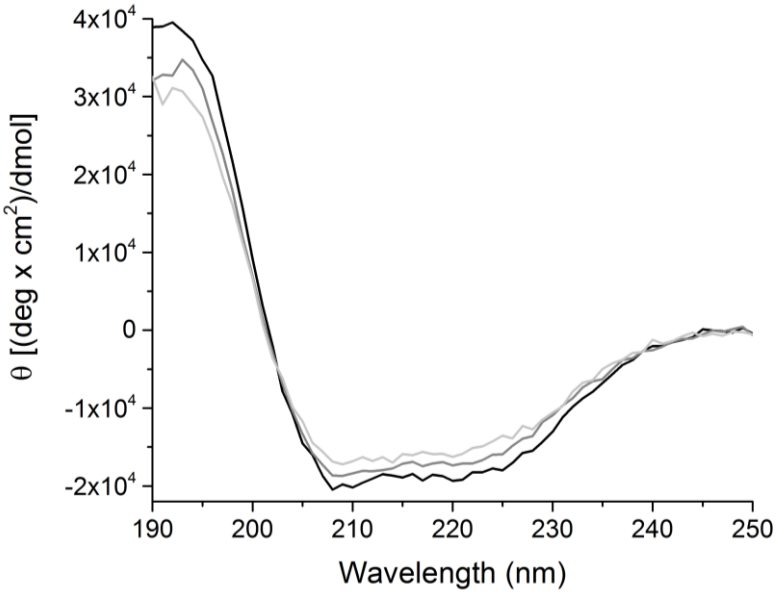


**Figure 4.7: CD spectra of bovine serum albumin (BSA) in water (–), ethanol-pretreated (EtPT) BSA in water (–) and BSA in 50% (v/v) ethanol (–).**

At room temperature, EtPT of soy glycinin increased the level of high MW compounds extractable in SDS medium (SE-profile not shown). After 6 min of heating, polymerization of soy glycinin was slightly slowed down after EtPT. Prolonged (>6 min) heating in water did not impact the overall SDS-EP content (Table 4.1). While soy glycinin unfolded into trimeric and hexameric complexes in 50%

(v/v) ethanol (Section 4.3.2), DSC analyses of EtPT soy glycinin in water showed only one single peak temperature (95.8 °C ± 0.4 °C) suggesting that the more thermostable hexameric conformation was again favored in water. However, EtPT of glycinin reduced the enthalpy and slightly decreased the denaturation temperature (Figure 4.2.C).

Moreover, EtPT impacted neither the MW distribution nor the SDS-EP content of gluten before or after prolonged (> 6 min) heating at 100 °C in water (Table 4.1). After 6 min of heating, polymerization of gluten was slightly increased as a result of EtPt. In conclusion, EtPT altered the conformation, denaturation and heat-induced polymerization at short times (≤ 6 min) of BSA and soy glycinin, but did not significantly impact the polymerization of proteins during prolonged heating in water at 100 °C.

Furthermore, it was investigated whether pretreatment of one protein type with aqueous ethanol affected its co-polymerization with other protein types. Gluten addition increased the denaturation temperature and enthalpy of BSA after EtPT (Figure 4.2.C). However, the stabilizing effect during heat-induced denaturation of gluten on BSA after EtPT was less than gliadin had on BSA without EtPT (Figure 4.2.B). However, EtPT of BSA did not impact the overall extractability loss of a BSA-gluten mixture during heating in water (Table 4.2).

**Table 4.2: Proteins extractable in sodium dodecyl sulfate medium (SDS-EP, in %, with standard deviations between brackets) of a mixture of bovine serum albumin (BSA) with gluten (ratio 1:2). Proteins with subscript EtPT have been pretreated with 50% (v/v) ethanol. The mixtures of the proteins have been heated for various times at 100 °C in water.**

| | SDS-EP (%) | | | |
|---|---|---|---|---|
| Sample ratio (1:2) | 0 min | 6 min | 60 min | 120 min |
| BSA/gluten | 86 (6)[a] | 28 (1)[a] | 14 (1)[a] | 14 (1)[a] |
| $BSA_{EtPT}$ /gluten | 83 (2)[a] | 28 (1)[a] | 16 (1)[a] | 16 (1)[a] |
| $BSA/gluten_{EtPT}$ | 78 (4)[a] | 22 (2)[b] | 10 (1)[b] | 10 (1)[b] |

**Results in the same column indicated with the same letter are not significantly different (α = 0.05).**

In contrast, pretreatment of gluten with 50% (v/v) ethanol increased the extractability loss of a BSA-gluten mixture during heating in water (Table 4.2). EtPT of gluten increased gliadin incorporation in the protein network (SE-profiles not shown). While the presence of BSA increased the extent of polymerization of gluten, gluten slowed down the extent of polymerization of BSA, especially after EtPT of gluten. After short heating times (2 min), SDS-extractable polymers eluting at 5 min 40 sec contained both BSA and gluten protein and pretreatment of gluten with 70% (v/v) ethanol increased their relative levels (Figure 4.8). Possible explanations for the increased extractability loss of a BSA-gluten mixture after EtPT of gluten include (i) conformational changes of gluten proteins, (ii) redistribution of lipids and (iii) release of gliadin from the glutenin network. Changes in glutenin

secondary structure due to heating have been related to changes in gluten physicochemical properties (hydrophobicity, SH- and SS content) (Weegels *et al.* 1994). Glycolipids are preferentially associated with glutenin while phospholipids tend to interact with gliadin and lipid binding proteins in gluten (McCann *et al.* 2009). The extraction of bound lipids with aqueous ethanol may redistribute lipids and thereby change the interactions between proteins. The EtPT of gluten also releases gliadin from the glutenin network, thereby increasing its opportunity to react with BSA.

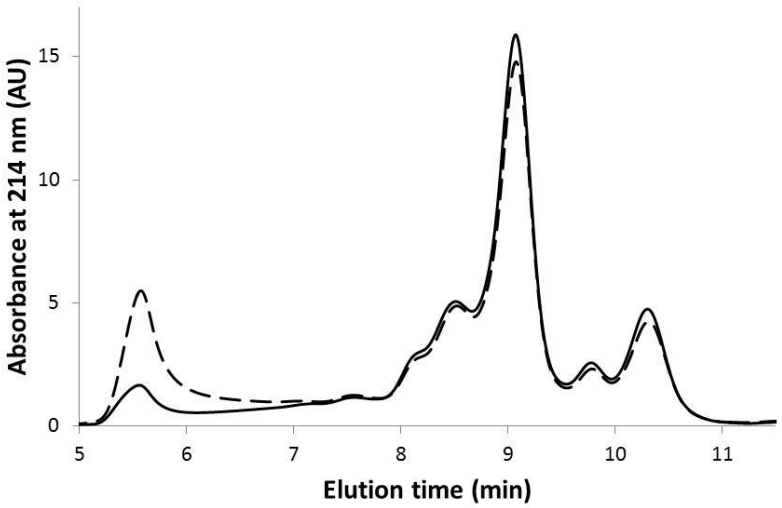


**Figure 4.8: SE-HPLC profile of protein extracts in sodium dodecyl sulfate medium (SDS-EP) of mixtures (1:2 ratio) of bovine serum albumin (BSA) and wheat gluten (–) or 70% (v/v) ethanol-pretreated wheat gluten (--) heated for 2 min at 100 °C in water. AU, arbitrary units.**

## 4.4 Conclusion

Already at room temperature, contact with ethanol impacts the MW distribution of some proteins. For instance, BSA aggregated in 50% (v/v) ethanol, not just due to non-covalent interactions but also due to SS cross-linking. Increasing the ethanol concentration reduces polymerization of soy glycinin and gliadin at 100 °C. Gliadin polymerization was even blocked in 50% (v/v) ethanol. Aqueous ethanol decreases the rate of β-elimination, SH oxidation and SH-SS exchange reactions. Furthermore, it alters protein conformation, availability of reactive groups and colloidal stability. In water but not in aqueous ethanol, gliadin-BSA and gliadin-soy glycinin mixtures polymerize more than expected based on polymerization of the isolated proteins under equal conditions. An important finding is that thermodynamic compatibility is not the key parameter enhancing covalent network formation between proteins. In complex aqueous model systems with or without ethanol, different protein types influence each other's polymerization, even when they are phase-separated. Pretreatment with aqueous ethanol alters protein conformations and denaturation properties but does not influence network formation during prolonged heating of isolated proteins. In contrast, pretreatment of gluten impacts heat-induced polymerization of BSA in gluten-BSA mixtures.

# Chapter 5

# Heat-induced polymerization of different globular food proteins in mixtures with wheat gluten

## 5.1 Introduction

As shown in Chapter 4, differences in solubility do not hinder the occurrence of positive co-protein effects, which is here referred to as more heat-induced polymerization than what would be expected based on the isolated proteins, in a mixture of BSA and gliadin in water. While the above points to the importance of co-protein effects in mixtures of gluten and globular proteins, which are common in some bakery, pasta and noodle products, the underlying mechanisms are poorly understood. It is especially important to investigate how proteins influence each other's functionality and which protein characteristics favor heat-induced polymerization. Fundamental knowledge on co-protein effects would be helpful to predict co-protein effects and can form a basis for developing new food products and recipes.

The aim of the present Chapter was to develop a model for predicting potential co-protein effects between gluten and globular proteins during heating at 100 °C. Boiling in water is relevant for food products such as pasta and noodles. To that end, the polymerization behavior (formation of SS bonds, LAN and LAL, changes in MW and extractability in SDS medium) of isolated food proteins was studied using five well-documented food proteins (BSA, soy glycinin, ovalbumin, S-ovalbumin and

lysozyme) varying in amino acid composition and structure. Next, heat-induced changes in mixtures of proteins derived from the same source were investigated, focusing on egg and wheat proteins. Finally, co-protein effects between the above mentioned proteins and gluten were analyzed and linked to specific protein characteristics. It was hypothesized that larger proteins having few ionized groups, high levels of accessible SH groups and hydrophobic patches would enhance the polymerization of gluten more than other proteins.

## 5.2 Materials and methods

### 5.2.1 Materials and characterization thereof

Different egg, soy and whey fractions have been studied in combination with wheat gluten. BSA, ovalbumin, S-ovalbumin, lysozyme and soy glycinin were selected as 'model proteins' with substantial differences in free SH and SS content, pI, and hydrophobicity. Furthermore, whole egg, egg white and egg yolk were studied because they are relevant for various food products. To investigate whether other wheat proteins can influence network formation of gluten, the albumin and globulin wheat fractions were also studied.

**Gluten** (83.2% protein on dm) was isolated form wheat flour cv Paragon (RAGT) as in Section 3.2.1. **Wheat albumins** (42.9% protein on dm) and **globulins** (69.1% protein on dm) were extracted (60 min, room temperature) from flour (50.0 g) with respectively 100.0 ml water or 100.0 ml sodium phosphate buffer (0.050 M; pH 7.6) containing 0.4 M sodium chloride. After three extractions with each medium and intermediate and final centrifugation (10 000 g, 10 min) steps, the combined water or buffered supernatants were dialyzed for 24 h against 0.01% acetic acid. **Gliadin** (87.7% protein on dm) was obtained by extraction with 70% (v/v) ethanol as in Section 4.2.1. The residue obtained after gliadin extraction was further extracted twice with 60% (v/v) ethanol. These extracts were discarded. Starch was then washed from the pellet with deionized water and sieving. The resultant residue was called **glutenin** (82.4% protein on dm). **Soy glycinin** (98.1% protein on dm) was isolated from soy flour (L.I. Frank) as in Section 3.2.1. Commercial **eggs** (55.6% protein on dm) were whipped with a whisk or separated in **egg white** (90.1% protein on dm) and **egg yolk** (33.6% protein on dm) with removal of the vitelline membrane. All protein fractions were freeze-dried and gently ground in a mortar. Lipids were removed from freeze-dried egg yolk (50.0 g) with hexane. After five extractions (each with 250.0 ml) and filtration, the **defatted egg yolk** (67.2% protein on dm) was air dried. **Hen egg lysozyme** (chicken egg white, 100.0% protein on dm) and **ovalbumin** (albumin chicken egg grade III, 94.1% protein on dm) were from Sigma-Aldrich. Ovalbumin (6.67 mg/ml) was shaken for 24 h at 55 °C in a glycine-sodium hydroxide buffer (0.10 M; pH 9.9) for converting it into **S-ovalbumin**. After

dialyzing for 24 h against deionized water, S-ovalbumin (91.6% protein on dm) was freeze-dried and ground. **BSA** (fraction V for biochemistry, 98.2% protein on dm) was from Acros Organics. **Whey protein isolate** (97.7% protein on dm) was from Fonterra (Amsterdam, The Netherlands). Moisture contents were determined in triplicate according to AACC-I Approved Method 44-15.02 (AACC 1999). Protein contents were determined in triplicate, with an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands) as in Section 3.2.2. Conversion factors (5.7 for gluten; 6.25 for all other proteins) were used to calculate protein from nitrogen contents. All chemicals were of analytical grade and from Sigma-Aldrich unless specified otherwise. DTT, disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International.

### 5.2.2 Heat treatment

Solutions or dispersions of the isolated proteins or mixtures thereof with gluten in deionized water (ratio 2:1 w/w, 100.0 mg protein/ml) were heated in hermetically sealed tubes at 100 °C for 0, 6, 60 and 120 min as in Section 4.2.3 at least in duplicate. Heat-treated sample tubes were immediately cooled by putting them in water. The pH of unheated samples was determined in duplicate after shaking for 60 min at room temperature. All samples were freeze-dried and ground.

### 5.2.3 Differential scanning calorimetry

DSC measurements were performed in triplicate as in Section 4.2.5 using aluminum pans (Perkin-Elmer).

### 5.2.4 Determination of surface hydrophobicity

The protein surface hydrophobicity was determined in duplicate with 1-anilino-8-naphthalene sulfonate (ANS). Unheated and heated (60 sec; 100 °C; in duplicate) proteins (10.0 mg protein/ml) were diluted with 0.01 M sodium phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.05 to 0.50 mg/ml. Samples (200 µl) were transferred to a 96-well plate and 10 µl ANS solution [8.0 mM in 0.01 M sodium phosphate buffer (pH 7.0)] was added. The fluorescence of the protein samples was measured with a Synergy MX Multi-Mode Reader (BioTek, Winooski, VT, USA). Wavelengths of excitation and emission were 390 nm and 480 nm, respectively. The protein surface hydrophobicity was calculated as in Chaudhuri *et al.* (1993). Hereto, the relative fluorescence was calculated as the difference in fluorescence intensity of the protein-ANS mixture and that of solution in buffer without protein (control ANS) and divided by the fluorescence of control ANS. The slope of the plot of relative fluorescence intensity as a function of protein concentration represents the protein surface hydrophobicity.

### 5.2.5 Determination of free sulfhydryl content

Free SH groups were determined colorimetrically with DTNB as in Section 4.2.6. Samples (1.0-1.5 mg protein) were shaken for 60 min at room temperature in 1.0 ml sample medium [0.10 mol/l tris(hydroxymethyl)aminomethane-HCl (pH 7.0) containing 2.0% SDS, 3.0 M urea and 1.0 mM tetrasodium ethylenediaminetetraacetate]. Then, 100 µl of DTNB reagent [0.1% (w/v) in sample buffer] was added and the samples were shaken for 10 min. After filtration (Millex-HP, 0.45 µm, Merck Millipore), the absorbance at 412 nm was read exactly 55 min after addition of DTNB reagent. Extinction values were converted to concentrations of free SH groups using a calibration curve with glutathione. Controls without DTNB or sample were used to correct for background absorbance of DTNB and sample. All analyses were performed in triplicate. Based on the weight average of the experimental values of the corresponding isolated proteins an expected free SH content was calculated for mixtures of globular proteins with gluten (ratio 1:2 w/w).

### 5.2.6 Determination of monomeric particle size and ζ -potential

Duplicate samples (10.0 mg protein/ml) were extracted (60 min, room temperature) with 0.10 M NaCl. After centrifugation (5 000 *g*, 5 min) the particle size distribution of protein in the supernatant was determined using dynamic light scattering (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). In this method, the Brownian motion of particles is related to their size based on the Stokes-Einstein equation. Monomeric globular albumins and globulins are here represented as spheres. The ζ-potential was determined on unheated and heated (1 min; 100 °C) samples with particle electrophoresis (Zetasizer Nano ZS series). A short heating step was performed to unfold the globular proteins while minimizing polymerization. All measurements were carried out at 20 °C.

### 5.2.7 Size exclusion high performance liquid chromatography

The impact of heating on the extractability in SDS medium and the MW distribution of SDS extractable proteins was evaluated with SE-HPLC as optimized in Chapter 3. This method was repeatable for samples heated on different days. Samples (1.0 mg protein/ml) were extracted (60 min, room temperature) with SDS medium. Extraction under reducing conditions was performed under nitrogen atmosphere with SDS/DTT medium. All analyses were performed in triplicate. The total SDS-EP was calculated from the corresponding peak areas and expressed as a percentage of assumed total area, that is, the area recorded under reducing conditions. The expected SDS-EP values were calculated as the weight average of the experimental SDS-EP values of the corresponding isolated proteins.

### 5.2.8 Analysis of lysinoalanine, lanthionine and cystine

Amino acids, including cross-links as cystine, LAN and LAL, were analyzed with high-performance anion-exchange chromatography with pulsed amperometric detection. To that end, (iso)peptide bonds of samples (10.0 mg protein/ml) were hydrolyzed by heating at 110 °C for 24 h with 6.0 M HCl containing 0.1 % (w/v) phenol and 3.0 mM norleucine (as internal standard). Then, mixtures were diluted (800-fold) in deionized water, filtered (Millex-GP, 0.22 µm) and analyzed as in Rombouts *et al.* (2009) with some modifications. To avoid underestimation of the total amount of cysteine due to acid-induced degradation, cysteine and cystine were oxidized to cysteic acid prior to acid hydrolysis as in Moore (1962). Performic acid was formed by adding 1.0 ml 35% hydrogen peroxide to 9.0 ml formic acid and resting for 60 min at room temperature. The resulting performic acid containing solution was then cooled to 0 °C on ice. An aliquot (4.0 ml) was then added to the samples which were then left overnight (16 h, 0 °C). To remove the excess performic acid, 0.6 ml 48% hydrogen bromide was added. Bromine and formic acid were evaporated from the samples with a Rotational Vacuum Concentrator (Q-Lab) at 60 °C and 100 Pa, followed by amino acid analysis as described in Rombouts *et al.* (2009) with 0.15 mM norleucine as internal standard. Mixtures were diluted (200-fold) in deionized water before filtration and quantification of cysteic acid. All analyses were performed in triplicate. The expected levels of LAN and LAL were calculated as the weight average of the experimental values of the corresponding isolated proteins.

### 5.2.9 Kinetics of extractability loss in sodium dodecyl sulfate containing medium

In bread and pasta, the loss in extractability in SDS medium during heating is attributed to SH-SS exchange reactions. Such reactions, which can *e.g*. be initiated by glutenin by nucleophilic attack of a free SH group to an adjacent intramolecular SS bond of gliadin resulting in an SDS-unextractable gliadin-glutenin polymer, has been reported to proceed according to first-order kinetics (Lagrain *et al.* 2008a; Bruneel *et al.* 2011). Furthermore, BSA and mixtures of BSA with gluten lose extractability in SDS containing medium following first-order kinetics during heat treatment (Rombouts *et al.* 2012c). Protein extractability in SDS medium (y, expressed as SDS-EP) is assumed to decrease during heating towards a minimum and can be presented as follows:

$$-\frac{\mathrm{dy}}{\mathrm{dt}} = \mathrm{k[y]} \qquad \text{(Equation 5.1)}$$

with k the first-order reaction rate constant of loss in extractability in SDS medium ($min^{-1}$). Hence, with $[y]_0$ and $[y]_t$ the SDS-EP at time zero and time t respectively is given by

$$[y]_t = [y]_0 \, e^{-kt} \quad \text{(Equation 5.2)}$$

The protein extractability decreases towards a minimum during heating time according to the equation

$$[y]_t = [y]_0 \; e^{-kt} + [y]_{minimal} \quad \text{(Equation 5.3)}$$

with $[y]_{minimal}$ the extractability of protein resisting polymerization under the experimental conditions. Trend lines of protein polymerization according to first-order kinetics were estimated using JMP® Pro 11.2.0 (SAS Institute). Due to the limited heating time points, first-order kinetics were used in this Chapter to support the experimental data but no k, [y] or $R^2$ values are reported in the text.

### 5.2.10 Prediction model

To predict whether a food protein would experience a co-protein effect in the presence of gluten, a multiple linear regression model was developed which takes into account a number of experimentally determined protein characteristics [*i.e.* monomeric particle size, free SH content, cysteine level, denaturation temperature of the largest fraction, ζ-potential and surface hydrophobicity from the unheated and heated (1 min; 100 °C) samples]. For each heating time, the co-protein effect (in %) was calculated as the ratio of experimental to expected SDS-EP. A ratio higher, similar or lower than 100% reflects a positive, no or a negative co-protein effect respectively. The experimental protein characteristics of ten isolated protein (mixtures) were related to their corresponding co-protein effect ratio when mixed with gluten. Because only a limited amount of samples was available, main-effects-only regression models were considered. An "all possible subsets" model selection procedure was executed using the Akaike Information Criterion (AIC) (Akaike 1973) and Adjusted $R^2$ as statistics to define the best model (Kutner *et al.* 2005). In this final model all terms significantly contributed to the prediction capability. These steps were performed using the JMP® Pro 11.2.0 software.

## 5.3 Results and discussion

### 5.3.1 Cross-linking of different isolated food proteins

Solutions of five isolated proteins were shaken for various times (0, 6, 60 and 120 min) at 100 °C in water. Their DSC peak denaturation temperatures were lower than 100 °C (Table 5.1).

**BSA** is the least heat-stable protein in this study (denaturation temperature *ca.* 62 °C). It has a larger portion of its hydrophobic residues at the surface than the other model proteins. Heating at 100 °C unfolds BSA exposing more negatively charged and less hydrophobic amino groups at the protein surface. BSA polymerizes rapidly during heating at 100 °C in water through SH oxidation and SH-SS

exchange reactions as described in Section 4.3.1, decreasing the SDS-EP of BSA to 3% ± 1% already after 6 min of heating (Figure 5.1.A). The level of its accessible free SH groups also rapidly decreased (6 min of heating) due to SH oxidation and slightly increased during extended heating (≥ 120 min) due to β-elimination reactions (Table 5.2).

**Ovalbumin** contains less cysteine (112 µmol/g protein) than BSA (528 µmol/g protein). However, one molecule of ovalbumin contains four free SH groups while a molecule of BSA contains only one such functional group (Tables 5.1 and 5.2). When ovalbumin unfolds, more of its negatively charged and hydrophobic amino acids become accessible at the protein surface (Table 5.1). During heating, the portion of free SH groups decreased (Table 5.2). The level of monomeric (eluting at *ca.* 9 min 5 sec) and dimeric (eluting at *ca.* 8 min 5 sec) ovalbumin decreased during heating with formation of polymers which remained extractable in SDS medium (eluting between 5 min 20 sec and 7 min 50 sec) and a low fraction of polymers not extractable in such medium (Figures 5.1.A and 5.2.1B). Only half of the accessible free SH groups of ovalbumin were consumed after 120 min heating (Table 5.2).

**S-Ovalbumin**, the more thermo-stable form of ovalbumin (Table 5.1), already polymerized during its production (SDS-EP of 84% ± 2%). It contained less accessible free SH groups than ovalbumin (Table 5.2). Also, residual buffer present as a result of the S-ovalbumin production could cause a higher pH reading of the S-ovalbumin solution than that of the ovalbumin solution (Table 5.1). SH groups ($pK_a$ 8.3) are more reactive under mild alkaline conditions than at lower pH (Visschers and de Jongh 2005). With a similar pI and a higher pH, S-ovalbumin was more negatively charged than ovalbumin (Table 5.1). More hydrophobic groups were already exposed in unheated S-ovalbumin than in ovalbumin but the increase during heating was lower. After 6 min of heating, monomeric and dimeric S-ovalbumin were converted to polymers which were either extractable or unextractable in SDS medium. SS bond formation (Figure 5.1.A, profile not shown) was crucial in this context. S-Ovalbumin formed polymers which were not extractable in SDS-containing medium in water to a larger extent than did ovalbumin.

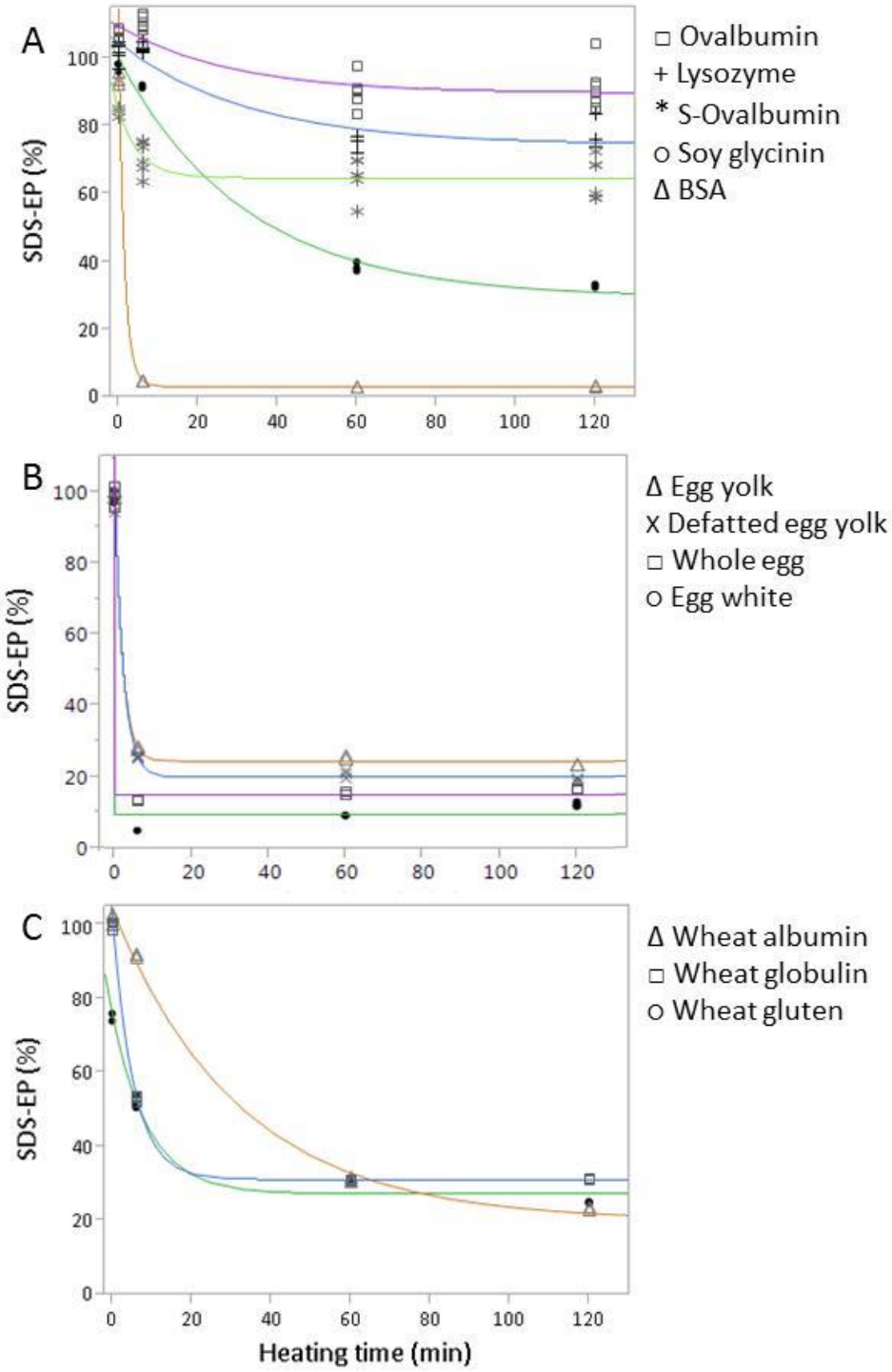


**Figure 5.1: Protein extractability in sodium dodecyl sulfate medium (SDS-EP) of different proteins [A, bovine serum albumin (Δ), hen egg ovalbumin (□), S-ovalbumin (∗), lysozyme (+) and soy glycinin (○)], hen egg [B, whole egg (□), egg white (○), egg yolk (Δ) and defatted egg yolk (x)] and wheat [C, gluten (○), albumin (Δ) and globulin (□)] fractions heated for various times at 100 °C in water. Trend lines were constructed assuming first-order kinetics.**

**Table 5.1: The pH of solutions/dispersions of unheated globular proteins (100.0 mg protein/ml), size of monomeric particles, total cysteine contents, peak denaturation temperatures ($T_d$), ζ-potential and surface hydrophobicity of different food proteins [bovine serum albumin (BSA), hen egg ovalbumin, S-ovalbumin, lysozyme and soy glycinin] and protein mixtures [hen egg (whole egg, egg white and defatted yolk) and wheat fractions (albumin, globulin)]. Samples were also heated for 60 sec at 100 °C to determine the ζ-potential and surface hydrophobicity. Standard deviations are between brackets.**

| Proteins | pH | Monomeric particle size (nm) | Cysteine (µmol/g) | $T_d$ (°C) | | ζ-potential (mV) | | Surface hydrophobicity $(mg/ml)^{-1}$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Peak 1 | Peak 2 | Unheated | Heated | Unheated | Heated |
| BSA | 6.9 (0.1) | 7.4 (0.4) | 528 (27) | 61.9 (0.3) | n.a. | -8.6 (0.1) | -13.1 (2.0) | 128.4 (9.8) | 109.8 (2.6) |
| Glycinin | 6.3 (0.1) | 11.6 (0.2) | 112 (8) | 96.8 (0.4) | n.a. | -5.6 (1.1) | -10.2 (1.9) | 0.2 (0.1) | 0.7 (0.2) |
| Ovalbumin | 7.3 (0.1) | 5.7 (0.2) | 252 (13) | 80.2 (0.1) | n.a. | -9.6 (1.7) | -15.8 (1.8) | 7.7 (0.7) | 88.8 (5.6) |
| S-Ovalbumin | 9.5 (0.1) | 5.7 (0.4) | 150 (24) | 91.1 (0.6) | n.a. | -11.5 (0.3) | -13.2 (0.5) | 16.9 (2.6) | 45.9 (3.8) |
| Lysozyme | 3.5 (0.0) | 3.3 (0.0) | 724 (14) | 73.0 (0.1) | n.a. | 9.8 (0.2) | 9.8 (2.8) | 0.8 (0.1) | 1.2 (0.1) |
| Whole egg | 9.1 (0.1) | 7.9 (1.7) | 265 (52) | 67.6 (0.3) | 81.6 (0.2) | -12.9 (0.1) | -14.5 (0.8) | 6.8 (0.1) | 45.9 (0.1) |
| Egg white | 10.0 (0.1) | 6.5 (0.4) | 238 (4) | 66.9 (0.1) | 80.5 (0.1) | -13.4 (0.2) | -13.5 (1.9) | 6.7 (0.8) | 76.1 (1.9) |
| Defatted egg yolk | 5.9 (0.1) | 6.9 (1.2) | 174 (11) | 74.1 (0.7) | n.a. | -6.9 (1.3) | -8.2 (0.3) | 1.6 (0.4) | 4.6 (0.2) |
| Wheat albumin | 6.0 (0.1) | 6.6 (1.6) | 322 (15) | 67.3 (0.1) | 89.1 (0.8) | -1.8 (0.1) | -1.8 (0.3) | 5.9 (0.1) | 11.1 (0.7) |
| Wheat globulin | 6.1 (0.1) | 10.8 (2.1) | 353 (6) | 64.0 (0.1) | 84.3 (0.3) | -2.9 (0.2) | -3.3 (0.7) | 5.6 (0.5) | 18.3 (0.8) |

n.a., not applicable

**Lysozyme** is positively charged in water and has low surface hydrophobicity (Table 5.1). This small protein with four SS bonds did not extensively polymerize after 6 min of heating at 100 °C (Figures 5.1.A and 5.2.1C). Prolonged heating (≥ 60 min) increased the level of free SH groups through β-elimination reactions (Table 5.2) which initiated polymerization (Figures 5.1.A and 5.2.1C). Eventually, lysozyme formed more polymers which were not extractable in SDS medium than did ovalbumin.

**Table 5.2: Free sulfhydryl (SH) contents of food proteins [bovine serum albumin (BSA), hen egg ovalbumin, S-ovalbumin, lysozyme and soy glycinin] and protein mixtures [hen egg (whole egg, egg white and defatted yolk) and wheat fractions (gluten, albumin, globulin)] heated at 100 °C in water for various times. Standard deviations are between brackets.**

| Proteins | Free SH content (µmol/g) | | | |
|---|---|---|---|---|
| | 0 min | 6 min | 60 min | 120 min |
| BSA | 7.7 (0.3) | 1.0 (0.2) | 1.3 (0.1) | 2.1 (0.1) |
| Ovalbumin | 62.0 (1.0) | 50.6 (0.5) | 40.5 (1.4) | 30.5 (1.3) |
| S-Ovalbumin | 29.9 (0.6) | 15.3 (0.8) | 5.3 (0.2) | 7.1 (0.3) |
| Lysozyme | 0.0 (0.3) | 0.0 (0.2) | 0.3 (0.1) | 1.1 (0.1) |
| Soy glycinin | 1.4 (0.3) | 0.3 (0.1) | 0.1 (0.2) | 0.0 (0.1) |
| Whole egg | 27.3 (0.8) | 1.2 (0.2) | 0.6 (0.1) | 0.4 (0.1) |
| Egg white | 32.6 (1.3) | 0.9 (0.1) | 2.0 (0.1) | 2.2 (0.1) |
| Egg yolk | 9.4 (0.6) | 3.6 (0.3) | 1.4 (0.5) | 1.5 (0.2) |
| Defatted egg yolk | 11.5 (0.5) | 1.0 (0.5) | 0.1 (0.4) | 0.1 (0.4) |
| Wheat gluten | 2.4 (0.1) | 0.0 (0.3) | 0.0 (0.2) | 0.3 (0.2) |
| Wheat albumin | 6.3 (0.1) | 0.0 (0.1) | 0.1 (0.1) | 0.8 (0.1) |
| Wheat globulin | 2.7 (0.2) | 0.0 (0.5) | 0.5 (0.1) | 0.0 (0.1) |

**Soy glycinin** is a large protein. It has a lower cysteine density than the other used model proteins (Table 5.2). During heating, the ζ-potential of soy glycinin solutions decreased and some hydrophobic patches were exposed. This protein polymerized faster and to a larger extent than lysozyme (Figure 5.1.A). A small portion of free SH groups formed during isolation initiated covalent network formation (Table 5.2, Figure 5.1.A). In agreement with Section 4.3.1, polymers extractable in SDS medium were formed after 6 min of heating which polymerized further to polymers unextractable in this medium during prolonged heating (Figure 5.2.1D).

Although ovalbumin rapidly initiated SH oxidation as a result of it containing four free SH groups per molecule, no continuous protein network was formed (Figure 5.2.1B). Even lysozyme, which needed β-elimination reactions to initiate SH oxidation and SH-SS exchange reactions, formed more polymers which were not extractable in SDS medium than ovalbumin (Figure 5.1.A). Free SH groups are required to initiate polymerization. However, these data indicate that the accessibility of intramolecular SS bonds also impacts the extent and continuity of the covalent network. For

example, BSA (1 SH group, 17 SS bonds) formed larger polymers which were unextractable in SDS medium than did ovalbumin (4 SH groups, 1 SS bond).

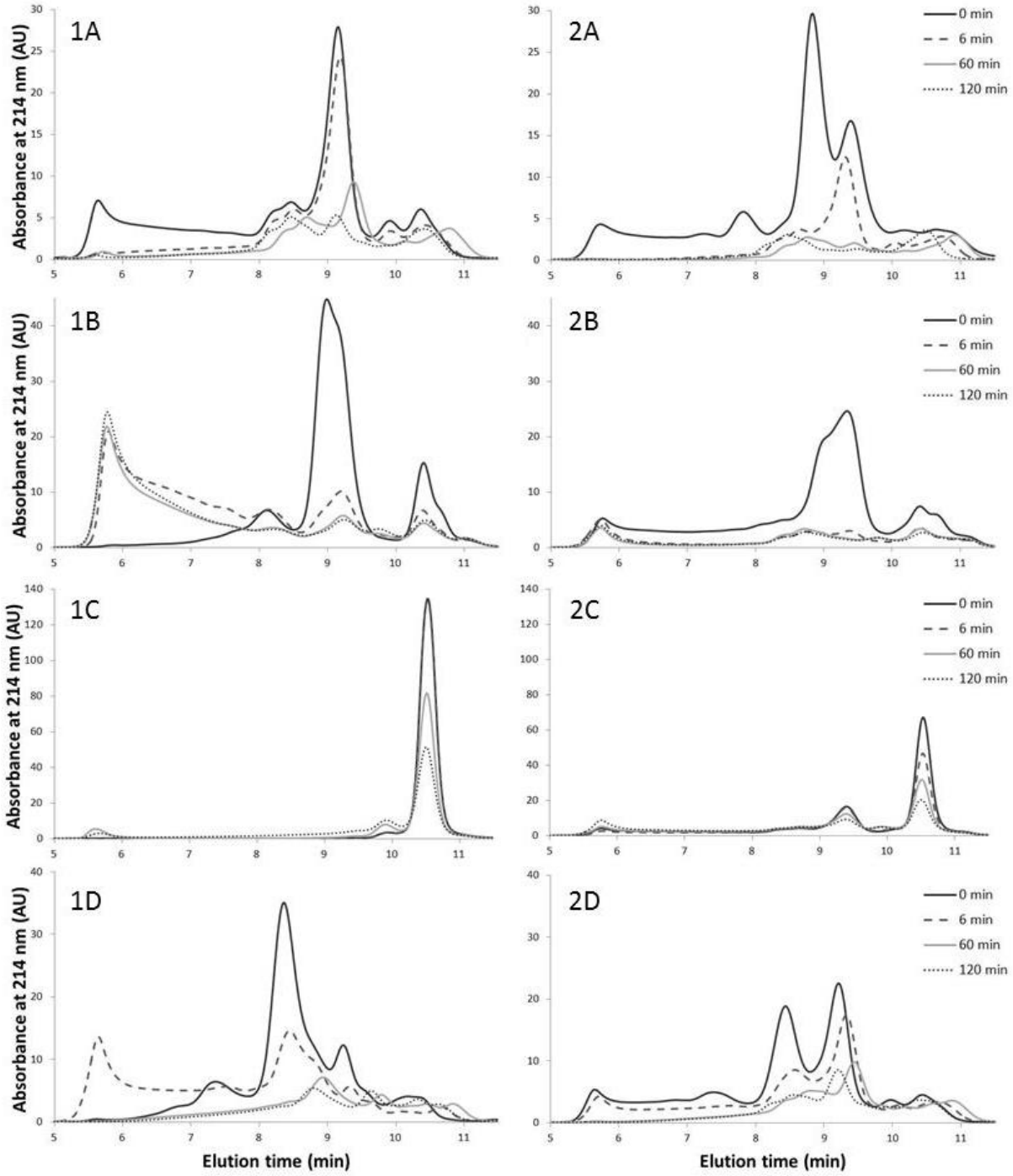


**Figure 5.2: SE-HPLC profiles of protein extracted in sodium dodecyl sulfate (SDS) medium of different proteins and mixtures thereof with wheat gluten (ratio 1:2 w/w). Wheat gluten (1A), hen egg ovalbumin (1B), lysozyme (1C), soy glycinin (1D) and mixtures of wheat gluten with bovine serum albumin (2A), hen egg ovalbumin (2B), lysozyme (2C) and soy glycinin (2D) before and after heat treatment at 100 °C for 6, 60 and 120 min in water. AU, arbitrary units.**

### 5.3.2 Cross-linking of egg proteins

The intrinsic characteristics and cross-linking of **egg white** were compared to those of its most abundant protein, *i.e*. ovalbumin. The pH of unheated lyophilized egg white solution was higher and its ζ-potential lower than those of a solution of ovalbumin of the same concentration (100.0 mg/ml) (Table 5.1). Heating for 60 sec at 100 °C increased the surface hydrophobicity in both samples, but slightly more in ovalbumin than in egg white. Egg white proteins formed more rapidly and to a larger extent polymers which were unextractable in SDS medium than did isolated ovalbumin (Figures 5.1.A and 5.1.B). Indeed, at 100 °C isolated ovalbumin formed more polymers that were still extractable in such medium, while egg white proteins immediately formed a network to an extent that they were no longer extractable in it. Ovalbumin is the only egg white protein which contains free SH groups (Huopalathi *et al.* 2007). Isolated lysozyme, which does not contain free SH groups, barely formed polymers which were not extractable in SDS medium (Figure 5.1.A). Even without concrete information on the extractability loss of the egg white proteins other than ovalbumin and lysozyme, it is undisputable that more polymers were formed in egg white which were not extractable in SDS medium than what could be expected based on data for the isolated proteins. The level of free SH groups in egg white decreased more rapidly than expected based on the loss of free SH in ovalbumin (Table 5.2). The formation of polymers not extractable in SDS medium in heated egg white implies that SH-SS exchange reactions occurred between ovalbumin and other egg white proteins containing more intramolecular SS bonds than ovalbumin. These results indicate that ovalbumin rapidly initiated polymerization in egg white thereby interconnecting other egg white proteins and leading to a continuous protein network. To the best of the authors' knowledge, the role of egg white proteins other than ovalbumin as network formers to date has been overlooked in the relevant food science literature.

In contrast to egg white, **egg yolk** solids contain *ca.* 62.5% lipids next to *ca.* 33% protein (Powrie and Nakai 1985). Prior defatting of egg yolk with hexane increased the extent of polymerization upon subsequent heating (Figure 5.1.B). Prior removal of lipids increased the opportunity of egg yolk proteins to react and the loss of free SH groups upon heating (Table 5.2). Egg yolk proteins polymerized to a smaller extent upon heating than egg white proteins even after defatting (Figure 5.1.B). Defatted egg yolk proteins also contained less cysteine and were less charged and hydrophobic at the protein surface than egg white proteins (Table 5.1).

The loss of SDS-EP in **whole egg**, which contains egg white and egg yolk in a ratio 2:1 (Powrie and Nakai 1985), equaled the sum of those of its fractions (Figure 5.1.B). There was hence no indication of a synergistic effect between egg white and yolk proteins.

### 5.3.3 Cross-linking of wheat proteins

**Wheat gluten** proteins contain high levels of glutamine and proline and low levels of charged amino acids. Even if cysteine is a minor amino acid in gluten, it largely impacts the structure of wheat-based food products (Wieser 2007). Before heating, 75% ± 2% of wheat gluten was extractable in SDS medium (Figure 5.1.C). As a result of heating, the extractability of glutenin (eluting between *ca.* 5 min and 7 min 55 sec) in this medium decreased more rapidly than that of gliadin (eluting between *ca.* 7 min 55 sec and 9 min 40 sec) (Figure 5.2.1A). Glutenin and gliadin polymerized about ten times faster during heating at 100 °C in water in each other's presence than absence (results not shown). Isolated glutenin polymerized fast during the first minutes of heating. At longer heating times (≥ 60 min), increased polymerization in gluten was attributed to increased gliadin incorporation. **Wheat albumin and globulin** were fully extractable before heating (Figure 5.1.C). The former polymerized more slowly than the latter, but reached a lower plateau value in terms of extractability in SDS medium. All wheat fractions had little free SH groups which rapidly oxidized into SS bonds during heating (Table 5.3). However, wheat gluten contained less cysteine (200 ± 7 µmol cysteine/g) than wheat albumin (322 ± 15 µmol/g) and globulin (353 ± 6 µmol/g) (Table 5.1).

### 5.3.4 Cross-linking between wheat gluten and (mixtures of) other food proteins

In some food products wheat gluten and other types of protein co-exist. Here, gluten was mixed with different globular proteins (ratio 2:1 w/w) and potential co-protein effects were monitored. The heat-induced SDS-EP losses of protein mixtures of wheat gluten with either BSA, ovalbumin or S-ovalbumin were larger than expected based on the SDS-EP losses of the isolated proteins (Figures 5.3.A, 5.3.B and 5.3.C). Such synergistic network formation is referred to as a positive co-protein effect. In the case of BSA (Figure 5.3.A), the difference between measured and expected SDS-EP readings was mainly due to increased gliadin incorporation (Figure 5.2.2A). In addition, gluten increased the denaturation temperature of BSA to 79.3 °C ± 0.3 °C, in agreement with findings by Rombouts *et al.* (2012c). Mixtures of ovalbumin and S-ovalbumin with gluten also polymerized faster and to a larger extent than expected based on observations for the isolated proteins (Figures 5.3.B and 5.3.C). After 6 min of heating, more gliadin was incorporated in the protein network of mixtures of gluten with (S-)ovalbumin than with BSA (Figures 5.2.2A and 5.2.2B). The ovalbumin co-protein effect with gluten was more pronounced than that of BSA (Figures 5.3.A, 5.3.B and 5.3.C). Gliadin lacks free SH groups. As a result, the presence of other proteins with free SH groups can initiate SH-SS exchange reactions and thereby substantially enhance protein cross-linking. The free SH content of (S-)ovalbumin-gluten mixtures was lower than expected based on observations for the isolated

proteins after 6 min of heating (Table 5.3) implying increased SH oxidation in the mixture. A negative co-protein effect was noted for the lysozyme/gluten mixture. The extractability loss of lysozyme-gluten mixtures was lower than expected based on the isolated proteins (Figure 5.3.D). After 6 min of heating, more free SH groups were accessible in this mixture with gluten than expected based on the isolated proteins (Table 5.3), and gliadin was not yet incorporated into the protein network (Figure 5.2.2C). As mixtures of equal weight basis were used, more protein molecules were present in lysozyme-gluten than in the other mixtures. The formation of a continuous protein network evidently requires more covalent cross-linking between smaller than between larger proteins. Thus, besides the absence of free SH groups, the small MW (*ca*. 14 kDa) could explain the negative co-protein effect. Soy glycinin showed no co-protein effect with gluten. The SDS-EP loss during heating was as expected based on that of the isolated protein (Figure 5.3.E). As soy glycinin has a high MW (*ca.* 360 kDa), the low amount of free SH groups seemed to be the factor limiting the triggering of a co-protein effect with gluten. In contrast with the isolated proteins, gluten contains sufficient intramolecular SS bonds to form a continuous network.

**Table 5.3: The pH of solutions/dispersions of unheated food proteins [bovine serum albumin (BSA), hen egg ovalbumin, S-ovalbumin, lysozyme and soy glycinin] and protein mixtures [hen egg (whole egg, egg white and defatted yolk) and wheat fractions (gluten, albumin, globulin)] with wheat gluten (100.0 mg protein/ml). Free sulfhydryl (SH) and lanthionine (LAN) contents of proteins with wheat gluten (ratio 1:2 w/w) heated at 100 °C for respectively 6 and 120 min. The expected values are the weight averages of the experimental SH and LAN contents of the corresponding isolated proteins. Standard deviations are between brackets.**

| Proteins with gluten | pH | Free SH groups (µmol/g) | | LAN (µmol/g) | |
|---|---|---|---|---|---|
| | | Experimental | Expected | Experimental | Expected |
| BSA | 6.9 (0.1) | 0.5 (0.0) | 0.1 (0.2) | 0.0 (0.0) | 5.0 (1.1) |
| Soy glycinin | 6.1 (0.1) | 0.6 (0.2) | 0.0 (0.2) | 0.0 (0.0) | 0.0 (0.0) |
| Ovalbumin | 6.4 (0.1) | 11.8 (0.7) | 16.8 (0.3) | 4.5 (1.3) | 7.7 (0.5) |
| S-Ovalbumin | 8.9 (0.1) | 2.7 (0.3) | 9.8 (0.3) | 8.9 (3.2) | 9.4 (5.0) |
| Lysozyme | 4.9 (0.1) | 1.7 (1.1) | 0.0 (0.2) | 0.0 (0.0) | 0.0 (0.0) |
| Whole egg | 7.1 (0.1) | 4.7 (0.6) | 0.2 (0.2) | 4.9 (1.2) | 12.2 (0.3) |
| Egg white | 8.2 (0.1) | 1.7 (0.3) | 0.1 (0.2) | 26.9 (0.9) | 35.0 (2.2) |
| Egg yolk | 6.1 (0.1) | 4.1 (1.2) | 1.0 (0.2) | 0.6 (0.1) | 0.8 (0.1) |
| Defatted egg yolk | 5.5 (0.1) | 1.0 (0.2) | 0.1 (0.3) | 1.3 (1.2) | 0.7 (0.1) |
| Wheat albumin | 6.0 (0.1) | 1.4 (0.4) | 0.0 (0.2) | 0.0 (0.0) | 0.0 (0.0) |
| Wheat globulin | 6.1 (0.1) | 0.8 (0.2) | 0.0 (0.3) | 0.0 (0.0) | 0.0 (0.0) |

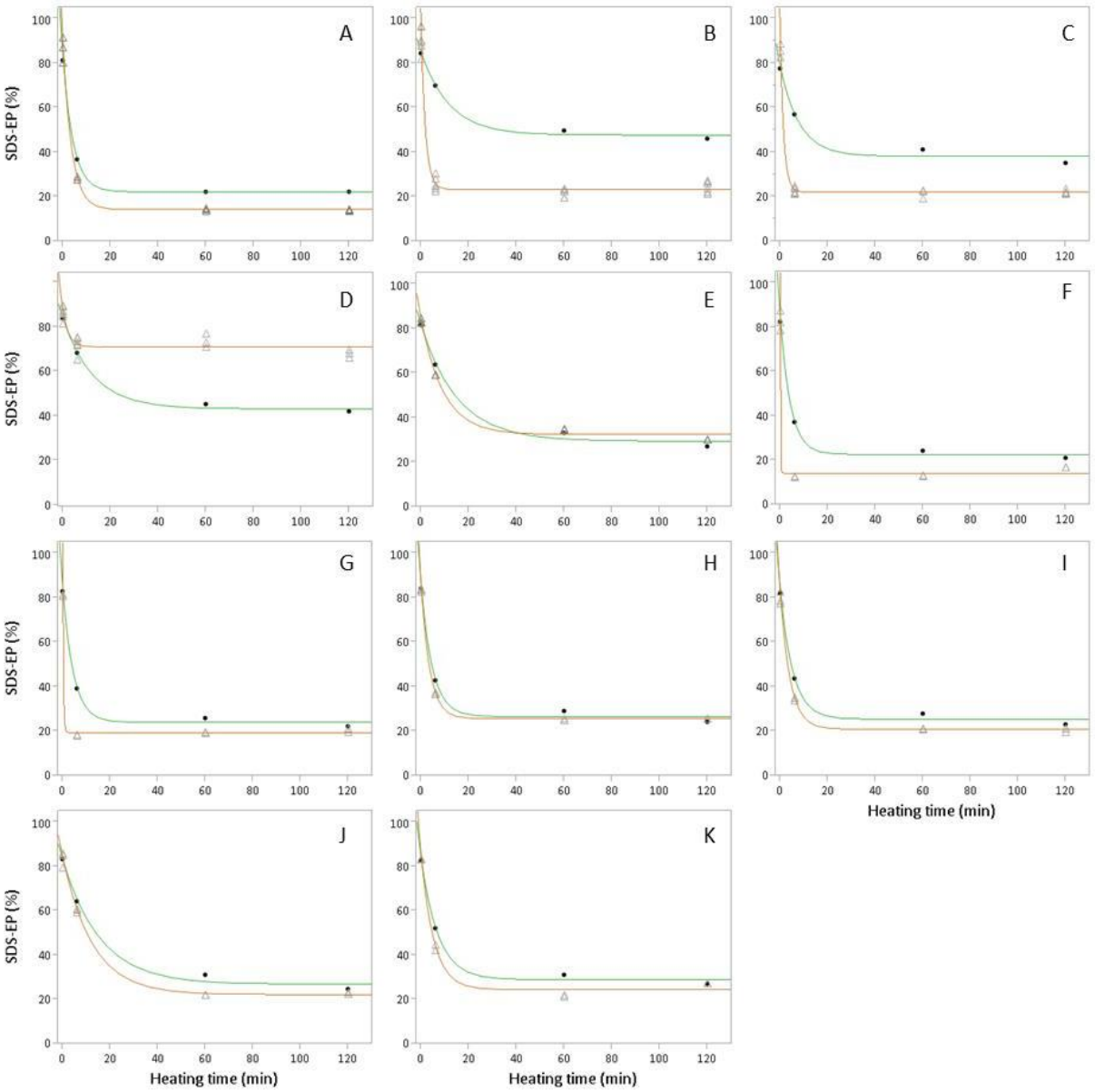


**Figure 5.3: Protein extractability in sodium dodecyl sulfate medium (SDS-EP) of mixtures of wheat gluten with bovine serum albumin (A), hen egg ovalbumin (B), S-ovalbumin (C), lysozyme (D), soy glycinin (E), hen egg white (F), whole egg (G), egg yolk (H), defatted egg yolk (I), wheat albumin (J) and wheat globulin (K). Results (Δ) are compared with values expected based on the isolated proteins (○). Trend lines were constructed assuming first-order kinetics.**

We also investigated the occurrence of co-protein effects of complex protein mixtures of one source with gluten. The protein mixtures were heated at 100 °C with gluten (ratio 1:2 w/w). Both egg white and whole egg showed a positive co-protein effect with gluten (Figures 5.3.F and 5.3.G). However, the difference between measured and expected SDS-EP was higher for egg white than for whole egg protein. Egg yolk lipids prevented the yolk proteins from exerting a co-protein effect with gluten (Figure 5.3.H). Indeed, defatted egg yolk proteins induced a co-protein effect with gluten (Figure 5.3.I). Furthermore, the measured SDS-EP of whole egg-gluten mixtures equaled the sum of the readings for egg white-gluten and egg yolk-gluten mixtures. Wheat albumin and globulin both

had a positive effect on protein network formation (Figures 5.3.J and 5.3.K). However, after 120 min of heating the polymerization of the mixtures was almost as expected based on the isolated proteins, because ω-gliadin and part of the albumin/globulin remained extractable (results not shown). So, even wheat proteins contribute to each other's polymerization. In all protein mixtures, less LAN was formed after 120 min heating than expected based on its formation in the isolated proteins (Table 3). After 120 min heating at 100 °C, LAL was formed neither in the isolated protein (fractions) nor in their mixtures with gluten. In these mixtures, SS bond formation seemed to be favored above other covalent reactions. While gluten increased the denaturation temperature of BSA largely, the impact of gluten on the denaturation temperatures of the other tested food proteins was less pronounced (results not shown). All globular proteins denatured during heating at 100 °C in mixtures with gluten.

To reveal which protein characteristics impacted the co-protein effects, the experimentally determined monomeric particle size, free SH content, cysteine level, denaturation temperature of the largest fraction, ζ-potential and surface hydrophobicity of unheated and heated samples of ten proteins (Tables 5.1 and 5.2) were linked to co-protein effects after 120 min of heating in the presence of gluten. Egg yolk was excluded as its lipids affected protein network formation. The resulting model describes whether the inclusion of specific globular proteins would induce a positive, no or negative co-protein effect in a mixture with gluten. Equation 5.4 was found with an $R^2$ of 0.79 (Figure 5.4):

$$\text{Co-protein effect}_{120\ min}\ (\%) = 85.00 + 0.82 \times \text{free SH content}\ (\mu mol/g) + 0.50 \times \text{surface hydrophobicity heated sample}\ [(mg/ml)^{-1}] \quad \text{(Equation 5.4)}$$

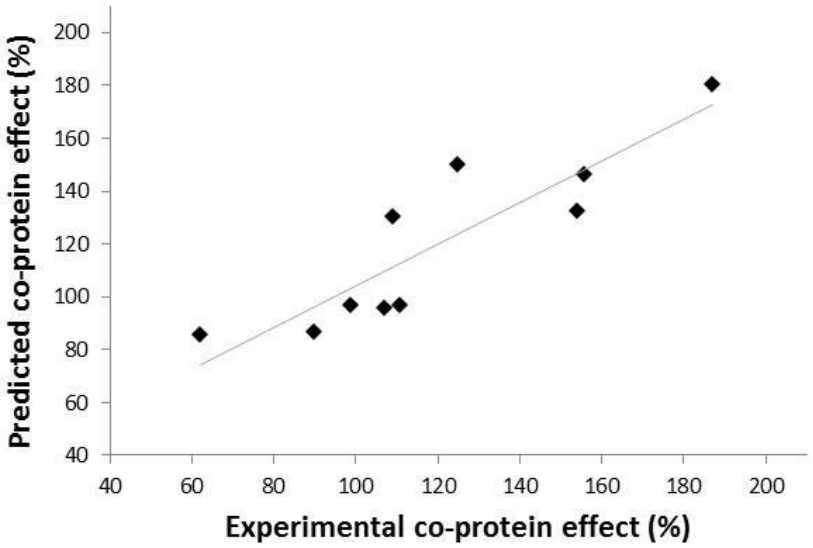


**Figure 5.4: Goodness of fit between the experimentally determined co-protein effects of globular proteins with gluten after 120 min of heating in water at 100 °C and the predicted co-protein effects based on Equation 5.4. Linear regression (in grey) revealed an $R^2$ of 0.79.**

The free SH content of unheated proteins and the surface hydrophobicity of heat-induced unfolded proteins were the main protein characteristics impacting co-protein effects in mixtures with gluten. Gluten contains little free SH groups. The accessible free SH groups of the added proteins play a key role because they can initiate SH-SS exchange reactions and thereby trigger the rapid formation of a protein network which involves both gluten and globular proteins. Furthermore, the model demonstrates the importance of non-covalent interactions for the formation of a covalent network in protein mixtures. When both protein types contain hydrophobic protein patches, these attractive forces reduce the distance between proteins and increase the opportunity for SH oxidation and SH-SS exchange reactions.

Applying this model for whey protein isolate, which had a free SH content of 25.4 µmol/g ± 0.3 µmol/g and a surface hydrophobicity value after heating of 19.6 $(mg/ml)^{-1}$ ± 1.5 $(mg/ml)^{-1}$, showed a slightly positive co-protein effect after 120 min of heating at 100 °C of 115.6%. Heating experiments with whey protein isolate and gluten showed an experimental co-protein effect of *ca.* 114.1%. These results confirm the validity of the here developed model to predict co-protein effects when heating globular proteins in combination with gluten in water at 100 °C. Indeed, while this model is based on an observational study with limited number of samples, it still seems a valuable tool to predict heat-induced polymerization of mixtures between wheat gluten on the one hand and any water or salt soluble globular protein on the other hand.

## 5.4 Conclusion

This work highlights the importance of some intrinsic protein characteristics for protein network formation, either in absence of other proteins, in the presence of proteins derived from the same source, or in the presence of proteins from a different source. For isolated globular proteins, key roles reside in the presence of free SH groups and SS bonds during heat-induced protein cross-linking. Accessible free SH groups initiate covalent network formation through SH oxidation and SH-SS exchange reactions. The levels of monomeric BSA (one free SH group) and ovalbumin (four free SH groups) decrease more rapidly than those of soy glycinin and lysozyme (no free SH groups). However, the polymers formed by (S-)ovalbumin remained largely extractable in SDS medium. It was hypothesized that the small amount of intramolecular SS bonds in ovalbumin hinders the formation of a continuous covalent protein network. Different proteins from one source can impact each other's polymerization. More polymers which are not extractable in SDS medium are formed in egg white than expected based on ovalbumin alone. Egg white proteins interconnect, which is referred to as a synergistic polymerization behavior. Even so, the extractability in SDS medium of whole egg proteins equals the sum of that of egg white and egg yolk proteins. In mixtures with gluten, a

negative co-protein effect when adding lysozyme, no co-protein effect when adding of soy glycinin and positive co-protein effects with BSA, ovalbumin and S-ovalbumin can be noted. The level of accessible free SH groups and the surface hydrophobicity of unfolded proteins are the main protein characteristics in terms of determining co-protein effects in mixtures with gluten.

# Chapter 6

# The role of wheat and egg constituents on protein network formation in egg noodles

## 6.1 Introduction

Using a model system approach, it was shown in Chapter 5 that different types of proteins can impact each other's network formation. Accessible free SH groups and hydrophobic patches of globular proteins enhance the polymerization with gluten proteins during heating at 100 °C in water. Co-protein effects can be of relevance for different food systems including cakes, cookies, pancakes, milk bread, pastry and egg noodles. The latter are made with little ingredients (wheat flour, egg and salt) and a straightforward production process (mixing, sheeting and slitting) which makes them ideal systems to study interactions between different proteins in real food products. Furthermore noodles are staple food in Asia with popularity all over the world. While the impact of eggs on the quality of wheat-based noodles and pasta has already been investigated, the impact of egg and gluten proteins on the rate and extent of protein network formation in fresh (raw) and cooked noodles is unknown.

The main objective of this Chapter was to study the functionality of wheat and egg constituents in fresh and optimally cooked noodles. First, the impact of protein quality and quantity and the role of different wheat Osborne-protein fractions on network formation and noodle properties were investigated. It was expected that noodles with higher gluten quantity and quality would have more

extensively developed protein networks. Second, the impact of whole egg, egg white and egg yolk on optimal cooking time, water absorption, cooking loss and noodle extensibility was determined. Third, the impact of egg constituents on actual protein network formation during noodle cooking was related to noodle properties. Based on the results of Chapter 5, it was hypothesized that the protein network in noodles containing whole egg would be better developed than noodles containing egg yolk but less developed than that in noodles containing egg white. Furthermore, noodles with a better developed network are expected to have superior cooking quality and strength. To study the impact of egg (fractions) and wheat proteins on protein network formation in noodles, covalent network formation was evaluated based on the loss of extractability in SDS medium. The influence of ionic interactions between proteins on noodle quality was studied by comparing noodles with and without salt addition. The impact of hydrophobic interactions and egg yolk lipids on noodle quality parameters and protein network formation was investigated by defatting egg yolk with hexane prior to noodle making. Defatting dried egg yolk with hexane removes mainly triacylglycerol, some phospholipids and cholesterol (Warren *et al.* 1988). Furthermore, olive oil was included in control noodle recipes (without egg fractions) and defatted egg yolk noodles to investigate the role of lipids. Finally, the role of hydrogen bonds between noodle constituents was outlined using low resolution proton nuclear magnetic resonance ($^{1}$H NMR).

## 6.2 Materials and methods

### 6.2.1 Materials and characterization thereof

Kernels from soft wheat cv Claire (Limagrain, Rilland, The Netherlands) and hard wheat cv Paragon (RAGT) were conditioned to 16.0% moisture and milled with a Bühler MLU-202 laboratory mill (Delcour *et al.* 1989) to **flour** with protein contents of 10.0% and 13.9% on dm respectively. **Wheat albumins** (36.9% on dm) and **globulins** (56.3% on dm) were sequentially extracted (60 min, room temperature) from wheat flour (50.0 g) with respectively water or sodium phosphate buffer (0.050 M; pH 7.6) containing 0.4 M sodium chloride (100.0 ml). After three extractions with each solvent and intermediate and final centrifugation (10 000 *g*, 10 min) steps, the combined supernatant of each solvent were dialyzed for 24 h against 0.01% acetic acid. Starch and **gluten** (Paragon gluten 79.7% protein on dm, Claire gluten 82.6% protein on dm) were isolated with a dough ball method as in Section 3.2.1. Commercial **eggs** (48.8% protein on dm) were whipped or separated into **egg white** (89.7% protein on dm) and **egg yolk** (33.6% protein on dm) with removal of the vitelline membrane. All protein fractions were freeze-dried and gently ground. Lipids were removed from freeze-dried egg yolk (50.0 g) with hexane (250.0 ml). After five repetitions of extraction and filtration, the **defatted egg yolk** (67.2% protein on dm) was air dried. Commercial olive oil (Bertolli,

extra virgin) was used. All chemicals were of analytical grade and from Sigma-Aldrich unless specified otherwise. DTT, disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International.

Moisture contents were determined in triplicate as in Section 5.2.1. Protein contents (N x 5.7 for wheat; N x 6.25 for egg protein) were determined in triplicate as in Section 3.2.2. The HMW-GS composition of wheat flour was determined based on Uthayakumaran and others (2006) using an Agilent (Agilent Technologies) LabChip of a protein 230 kit. HMW-GS were identified by comparing electrophoresis patterns of flour samples with known subunit compositions.

### 6.2.2 Noodle production and cooking

A typical egg noodle recipe includes 100 g flour (14% moisture content) and one fresh whole egg (*ca*. 48 g). To investigate the role of egg white and egg yolk in noodles, whole egg was replaced in the egg noodles recipe. Using lyophilized egg fractions, noodles were produced with constant protein content, moisture content, and ratio of flour to egg protein.

Salted control noodles were prepared from a quantity of wheat flour containing 86.0 g dm and 2.0 g salt. Such noodles made with flour of cv Claire and Paragon, further referred to as Claire and Paragon control noodles, had protein contents of 9.7% and 13.6% of dm and moisture contents of 33.1% and 32.2%, respectively. Whole egg, egg white or (defatted) egg yolk were added to obtain a moisture content equaling that of control noodles, a protein content of 14.6% and 18.0% of dm and a ratio flour to egg protein of 3:2 and 2:1 for Claire and Paragon noodles, respectively. To that end, starch and gluten from the respective wheat cv were added. Unsalted noodles prepared with flour of cv Paragon had protein and moisture contents of 18.7% of dm and 33.9%, respectively. In some experiments, olive oil was included in the recipe of unsalted control noodles and such noodles made with defatted egg yolk to obtain a noodle lipid content of 12.4% on dm. Furthermore, gluten, wheat albumin and wheat globulin were included in the recipes of unsalted control noodles to obtain the same protein content as when adding egg fractions.

The ingredients were mixed (5 min, 60 rpm) using a Kitchen Aid mixer (KPM5, St. Joseph, MI, USA) with intermediate scraping to include all ingredients adhering to the mixing bowl. After a dough rest of 30 min in a plastic bag at 23 °C, the dough was passed five times through a semi-automatic sheeter (Model C280 Capitani, Lomazzo Como, Italy) with a 2.9 mm roll gap with intermediate refolding. After compounding, the dough was rested a second time (30 min, room temperature) in a plastic bag. It was then sheeted once through 2.9 mm and each time twice through 2.1, 1.5, and 0.9 mm roll gap sizes successively. After each pass through the rolls, the dough was turned 180°. It was then cut (length 150 mm, width 5.0 mm) with a sheet cutter (Capitani).

Fresh noodle strands (20.0 g) were cooked in 500.0 ml deionized water to optimum as well as for 30 sec, 1, 3, 6, 12 and 20 min. The optimum cooking time was the minimum time needed to gelatinize all starch and determined as the point in time when an opaque core was no longer visible when squeezing the noodles between two glass plates according to the AACC-I Approved Method 66-55.01 (AACC 1999). Cooked noodles were immediately cooled in 200.0 ml deionized water at 23 °C. At least three different batches were made for each noodle type.

### 6.2.3 Noodle properties

Cooking loss was determined for each batch as the dm leached into the cooking water of optimally cooked noodles. Cooking and rinsing water were freeze-dried and the amount of dm was accurately weighed. Cooking loss (expressed in %) was calculated as:

$$CL\ (\%) = \frac{CL'(g,dm)}{FN\ (g,dm)}.100 \qquad \text{(Equation 6.1)}$$

with CL the cooking loss, FN the weight of fresh noodles and CL' the cooking losses on dm basis. The protein content (N x 5.7 for gluten noodles, N x 5.9 for other noodles) of cooking loss was determined with the Dumas method (section 2.1). Water absorption was determined for each batch and calculated by relating the weight increase between dry and optimally cooked noodles to the dm content of dry noodles with correction for the cooking losses as calculated by:

$$WA\ (\frac{g}{g\ dm}) = \frac{CN\ (g) - [FN\ (g) - CL'(g,dm)]}{[FN\ (g,dm) - CL'\ (g,dm)]} \qquad \text{(Equation 6.2)}$$

with WA the water absorption, CN the weight of optimally cooked noodles, FN the weight of fresh noodles and CL' the cooking loss on dm basis. The pH was determined in duplicate by suspending freeze-dried fresh noodle samples in deionized water (0.2 g/ml). It was measured after shaking (60 min, room temperature) with a pH meter HI 9025 (Hanna Instruments, Woonsocket, RI, USA). Fresh and cooked noodles were stretched with the Kieffer-rig dough and gluten extensibility rig (Stable Micro Systems, Surrey, UK) using an Instron (Norwood, MA, USA) 3342 with a 50 N load cell. After cooling (10 min, 23 °C), adhering water was gently removed from the surface of cooked noodles with paper. Ten individual noodle strands from each of two cooking batches were clamped between two plates and pulled upwards by a hook at 3.3 mm/sec until fracture. The maximum force during extension, the extensibility at breakage and the work needed to fracture, *i.e.* area under curve, were calculated from the force-displacement curves (Figure 6.1).

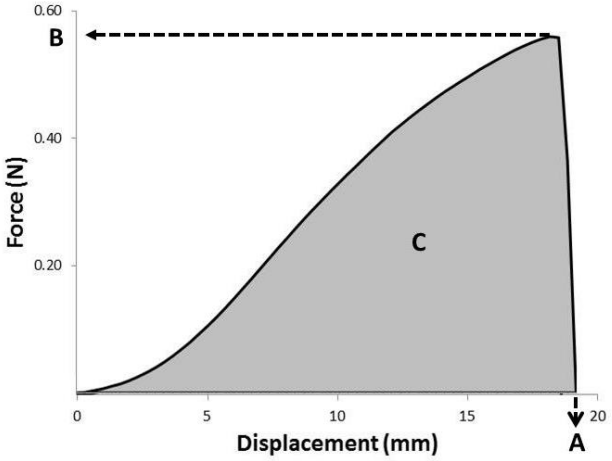


**Figure 6.1: A force-displacement curve of noodles using a Kieffer-rig dough and gluten extensibility rig. The extensibility at breakage (A), the maximum force during extension (B) and the total work needed to fracture (C, grey area) are indicated.**

### 6.2.4 Differential scanning calorimetry

DSC measurements were performed in triplicate on one batch as described in Section 4.2.5 with aluminum pans (Perkin-Elmer).

### 6.2.5 Low resolution proton nuclear magnetic resonance

Proton mobility distributions in fresh and cooked noodles were determined in triplicate on noodles of one batch as in Bosmans and others (2012) using a Minispec mq 20 low-field pulsed NMR spectrometer (Bruker, Ettlingen, Germany). After cooking, water on the noodle surface was gently removed with paper. The obtained transverse relaxation curves were fitted to a continuous distribution of spin-spin or transverse relaxation times ($T_2$) using the CONTIN algorithms of Provencher (Provencher 1982). Using the Free Induction Decay (FID) pulse sequence, less mobile proton populations were detected, whereas the Carr-Purcell-Meiboom-Gill (CMPG) pulse sequence was used to measure more mobile protons. The obtained peak areas are proportional to the amounts of protons in the population. Proton populations were assigned to noodle constituents based on starch-water, gluten-water, flour-water (Bosmans *et al.* 2012) and egg-water model systems (Luyts *et al.* 2013).

### 6.2.6 Size exclusion high performance liquid chromatography

The protein extractable in SDS medium and its MW distribution were determined as optimized in Chapter 3 in triplicate on noodles of one batch. The variance in noodles of one batch was similar to that between different batches. To evaluate extractability under reducing conditions, 0.10%, 0.25%, 0.50% or 1.00% (w/v) DTT was added to SDS medium under nitrogen atmosphere. Furthermore, a quantity of freeze-dried whole egg noodle containing 10.0 mg protein/ml was extracted sequentially with water and dimethyl sulfoxide (DMSO):propanol:water (ratio 2:1:1, v/v, hereafter referred to as

DMSO medium). After dilution (10x) with SDS medium, extracts were analyzed as described above. The total area of egg protein extracted in water and the areas of gluten protein extracted in DMSO medium, separated in glutenin, ω-, α- and γ-gliadin were integrated. About half of the gluten protein extractable in SDS medium was extractable in DMSO medium.

### 6.2.7 Kinetics of extractability loss in sodium dodecyl sulfate containing medium of cooked noodles

During heating, the extractability of gluten proteins in SDS containing medium decreases according to first-order kinetics as demonstrated for fresh pasta (Bruneel *et al.* 2011), bread (Lagrain *et al.* 2008a) and gluten-water model systems (Rombouts *et al.* 2012c). The extractability decreases towards a minimum but some protein remains extractable. The protein extractability in SDS medium (y, here expressed as the area in SE-HPLC chromatograms) is presented as in Section 5.2.9.

### 6.2.8 Statistical analysis

Significant differences ($\alpha = 0.05$), based on at least three measurements, were determined with the one-way ANOVA procedure using JMP® Pro 11.2.0 (SAS Institute). Corresponding Tukey grouping coefficients are given. Pearson correlation coefficients ($P < 0.05$) were calculated. Trend lines, corresponding equations and goodness of fit ($R^2$) of protein polymerization kinetics of cooked noodles were estimated on the basis of nonlinear regression analysis of the total extractable protein concentration as a function of heating time. Significant differences in equation parameters were determined with an equivalence test, comparing confidence intervals ($P < 0.05$).

## 6.3 Results and discussion

### 6.3.1 Importance of wheat protein network formation for noodle properties

Less protein was extractable in SDS medium from salted fresh Paragon noodles (SDS-EP control noodles 70% ± 4%) than from salted fresh Claire noodles (SDS-EP control noodles 84% ± 0%). Paragon gluten contains five HMW-GS including the subunits Ax1, Dx5 and Dy10 (Payne *et al.* 1981). This HMW subunit composition is positively related to noodle hardness and negatively to water absorption (Park *et al.* 2003) and indicates a superior gluten quality of Paragon than of Claire which contains the subunits Dx2, Bx7 and Dy12. After optimal cooking, comparable levels of their gluten protein were incorporated in the protein networks of salted Paragon (SDS-EP control noodles 27% ± 1%) and Claire (SDS-EP control noodles 30% ± 2%) noodles. However, salted Paragon noodles had higher protein content than Claire noodles. Paragon control noodles had higher optimal cooking times and absorbed significantly less water after optimal cooking than their Claire counterparts

(Table 6.1). These observations are in line with those of Park and Baik (2009) who found higher wheat flour protein contents to lead to lower water imbibition during noodle cooking and thereby to increase optimal cooking time. The cooking loss of salted Paragon control noodles was significantly lower than that of the corresponding Claire noodles (Table 6.1). In addition, Paragon fresh and cooked noodles showed significantly higher extensibility, maximum force, and total work during Kieffer-rig testing than did Claire noodles. The higher strength of Paragon than of Claire noodles can be ascribed to the better developed protein network in the former.

**Addition of wheat gluten to control noodles -** Addition of gluten to control noodle recipes increased the optimal cooking time of the resultant Paragon and Claire extra gluten containing noodles (hereafter referred to as gluten noodles) by 390 sec and 510 sec respectively. Park and Baik (2009) showed a higher reduction in water imbibition when including gluten in a noodle recipe for soft rather than in one for hard wheat. The impact of gluten addition on the Kieffer-rig parameters was higher in Claire than in Paragon fresh and cooked noodles (Table 6.1). Figure 6.2 shows the areas representing protein extracted in SDS medium, fitted according to first-order kinetics (Equation 5.3). Protein in gluten noodles polymerized slower (low k value) and more protein was incorporated in the protein network (lower $[y]_{minimal}$ value) than that in control noodles. Also, higher concentrations of DTT in the extraction medium were necessary to extract all protein from gluten noodles than from control noodles heated for 12 min at 100 °C (Figure 6.3). Thus, more protein was SS bound in gluten noodles than in control noodles. Furthermore, gluten noodles had higher protein content than control noodles. The higher protein level and degree of polymerization at optimal cooking time in gluten noodles led to more extensible noodles which required more work to fracture than control noodles (Table 6.1).

In unsalted fresh Paragon noodles, the extensibility at breakage and total work of control and gluten noodles was significantly higher than those in their salted counterparts (Tables 6.1 and 6.2). The addition of salt increased the time needed to develop wheat dough (Van Steertegem *et al.* 2013). However, no difference in SDS extractability was noticed between fresh noodles containing a range of salt concentrations (Rombouts *et al.* 2014). The optimum cooking time of unsalted noodles increased but the Kieffer-rig parameters after cooking were similar to that of salted noodles. Salt can shield ionic interactions. In fresh noodles, non-covalent interactions of the hydrogen bond type, as well as hydrophobic, ionic and Van der Waals interactions can impact Kieffer-rig parameters of noodles. During cooking, starch swells and proteins are covalently incorporated in the protein network. It is suggested that the swollen and gelatinized starch physically hinders non-covalent interactions between proteins and covalent cross-links become more important for the rigidity of the noodle structure.

**Table 6.1: Dough pH, optimal cooking time, water absorption, cooking loss, extensibility until fracture, maximum force and total work needed for fracture determined on fresh and optimal cooked salted noodles. Noodles with different hen egg proteins and wheat gluten were made with flour of soft wheat cultivar (cv) Claire and hard wheat cv Paragon.**

| Cv | Noodle type | Dough pH | Optimal cooking time | Water absorption (g/g dm) | Cooking loss (% on dm) | Extensibility (mm) | | Maximum force (N) | | Work (J) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fresh | Cooked | Fresh | Cooked | Fresh | Cooked |
| Claire | Control | 6.0 c | 5 min 30 sec | 2.76 c | 10.9 b | 10.5 d | 17.9 c | 0.47 b | 0.56 c | 2.8 c | 5.4 b |
| | Gluten | 6.1 c | 14 min | 3.02 bc | 9.9 b | 25.0 a | 30.6 a | 1.05 a | 0.74 b | 15.1 a | 13.1 a |
| | Whole egg | 7.0 b | 5 min 30 sec | 3.25 b | 9.3 b | 16.0 c | 24.7 b | 0.33 cd | 0.99 a | 2.8 c | 13.3 a |
| | Egg white[a] | 7.7 a | 9 min | 4.15 a | 14.0 a | 24.7 a | 20.3 c | 0.25 d | 0.72 b | 3.4 bc | 7.9 b |
| | Egg yolk | 6.3 c | 2 min 30 sec | 1.60 d | 6.0 c | 21.4 b | 11.5 d | 0.34 c | 0.43 d | 4.3 b | 2.3 c |
| Paragon | Control | 6.2 c | 6 min 30 sec | 1.96 bc | 6.5 b | 20.2 c | 26.7 c | 1.15 b | 0.92 b | 14.3 b | 13.6 c |
| | Gluten | 6.2 c | 13 min | 2.34 b | 6.8 b | 31.7 b | 37.5 a | 1.75 a | 1.15 a | 33.7 a | 24.6 a |
| | Whole egg | 6.9 b | 7 min | 2.02 bc | 6.3 b | 19.3 c | 32.3 b | 1.09 b | 1.24 a | 11.9 b | 22.5 ab |
| | Egg white[a] | 7.8 a | 13 min | 3.78 a | 11.3 a | 38.7 a | 27.0 c | 0.28 c | 1.22 a | 7.1 c | 19.1 b |
| | Egg yolk | 6.2 c | 3 min | 1.16 c | 4.4 b | 21.3 c | 18.2 d | 1.17 b | 0.79 b | 13.2 b | 7.7 d |

**Column values of one cv with the same letter are not significantly different (α = 0.05).**

**[a] Noodles with a higher moisture content of 37.1%.**

**Table 6.2: Optimal cooking time, water absorption, cooking loss and the portion of protein present in the cooking loss, extensibility until fracture, maximum force and total work for optimal cooked unsalted noodles made with different protein enriched Osborne-fractions of wheat flour cultivar Paragon.**

| Noodle type | Dough pH | Optimal cooking time | Water absorption (g/g dm) | Cooking loss (% on dm) | Protein in cooking loss (% on dm) | Extensibility (mm) | | Maximum force (N) | | Work (J) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fresh | Cooked | Fresh | Cooked | Fresh | Cooked |
| Control | 5.9 c | 9 min | 2.11 b | 5.4 b | 9.0 b | 29.1 c | 23.9 b | 1.19 b | 0.87 b | 23.0 b | 11.5 b |
| Wheat albumin | 6.7 a | 12 min 30 sec | 2.86 a | 11.6 a | 6.4 c | 39.8 b | 18.5 c | 0.90 c | 0.61 c | 25.1 b | 5.7 d |
| Wheat globulin | 5.6 d | 9 min | 2.42 ab | 6.4 ab | 15.7 a | 23.5 d | 19.6 c | 1.29 b | 0.86 b | 19.0 b | 8.5 c |
| Gluten | 6.0 b | 15 min 30 sec | 2.65 a | 5.6 b | 10.5 b | 50.3 a | 35.1 a | 1.78 a | 1.14 a | 60.1 a | 23.1 a |

**Column values with the same letter are not significantly different (α = 0.05).**

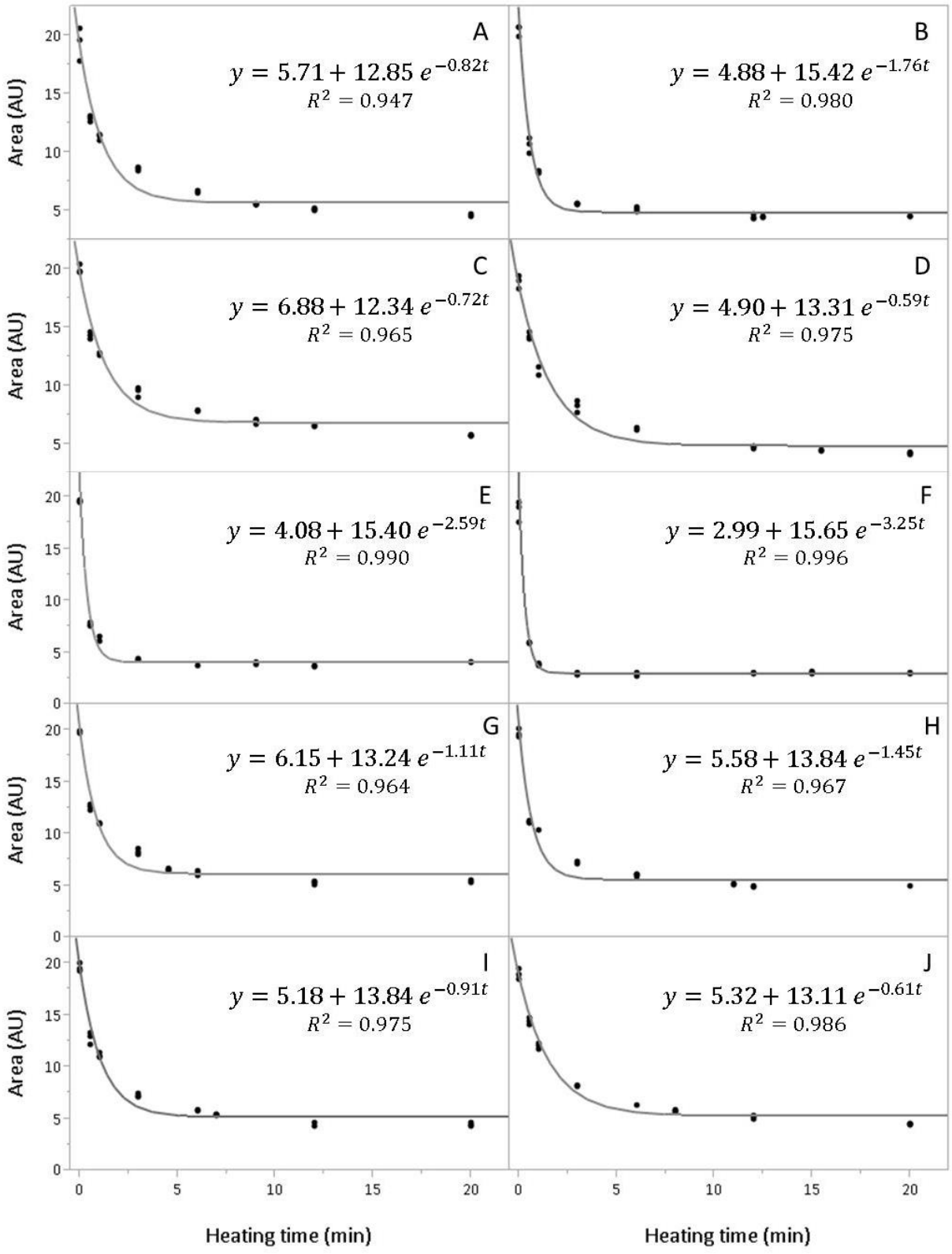


**Figure 6.2: Extractabilities (areas in SE-HPLC chromatograms) of protein in sodium dodecyl sulfate (SDS) medium from unsalted noodles made with flour of cultivar Paragon only (control noodles, A) and flour containing additional wheat albumin (B), wheat globulin (C), wheat gluten (D) hen whole egg (E), egg white (F), egg yolk (G), defatted egg yolk (H), defatted egg yolk with oil (I) and oil (J) after cooking at 100 °C. Trend lines and their corresponding formula, expressed as Equation 5.3, and goodness of fit ($R^2$) were achieved applying first-order kinetics. AU, arbitrary units.**

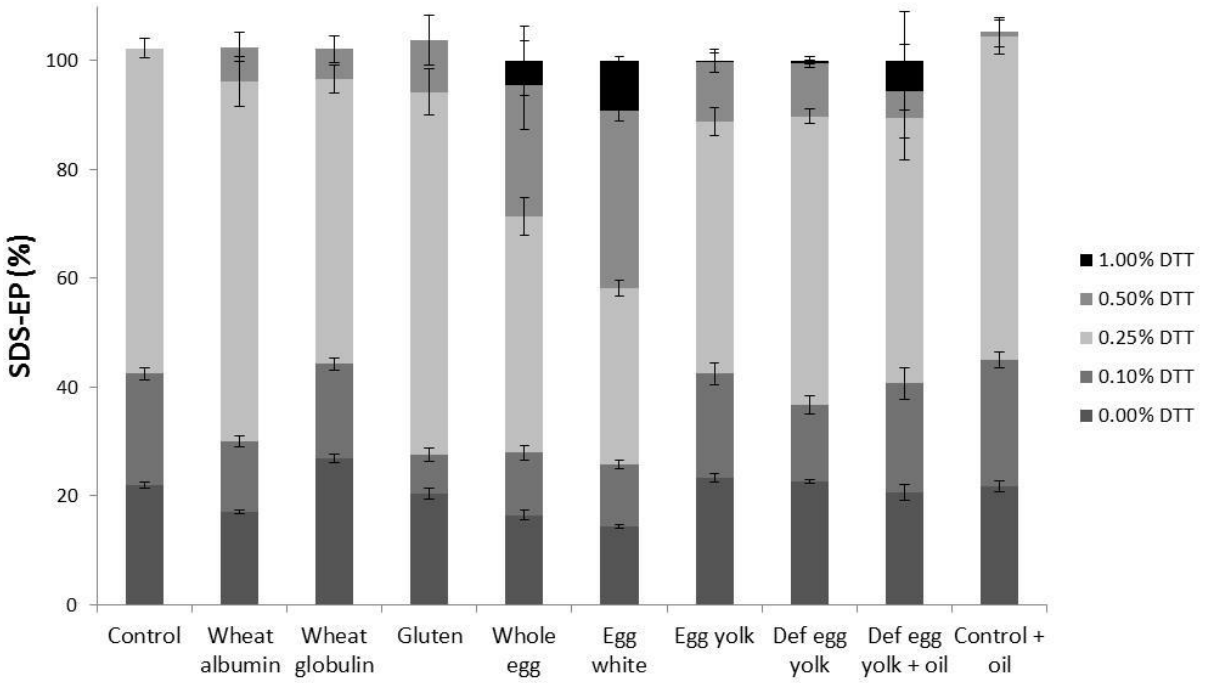


**Figure 6.3: Extractability of proteins in sodium dodecyl sulfate medium (SDS-EP) with increasing dithiothreitol (DTT) concentrations on unsalted noodles made with flour of cultivar Paragon only (control noodles) and flour with protein enriched Osborne-fractions of wheat, *i.e.* wheat albumin, wheat globulin and wheat gluten cooked and flour with hen whole egg, egg white, egg yolk, defatted (def) egg yolk, def egg yolk with oil and oil for 12 min at 100 °C. The SDS-EP is expressed as a percentage on the protein extractabilities of the corresponding samples in SDS/DTT medium. Standard deviations are given with error bars.**

**Inclusion of wheat albumin and globulin in control noodle recipe -** The inclusion of wheat albumin in the recipe of control noodles impacted the polymerization kinetics (Figures 6.2.A and 6.2.B). In the resultant wheat albumin noodles more protein was incorporated and interconnected by SS bonds than in control noodles (Figures 6.2.A, 6.2.B and 6.3). SE-HPLC profiles demonstrated that gliadin was more rapidly incorporated in the protein network in wheat albumin than in control noodles (results not shown). The optimal cooking time increased by 210 sec. The swiftly and extensively developed protein network in wheat albumin noodles did not withstand starch swelling which resulted in low cooking quality and low Kieffer-rig parameters after optimal cooking. When extra wheat globulin was included in the recipe, protein in the resultant wheat globulin noodles polymerized slower and less protein was incorporated in the network than in control noodles (Figures 6.2.A and 6.2.C). Also, more protein leached into the cooking water (Table 6.3). However, to extract all protein from wheat globulin noodles the SDS medium needed to contain at least 0.50% DTT while 0.25% DTT sufficed for control noodles (Figure 6.3). Less work was needed to stretch wheat globulin noodles than control noodles. Probably, protein network formation in wheat globulin noodles was insufficient to maintain noodle strength. The above results confirm that wheat albumin and globulin have a negative impact on noodle properties, even if their protein network formation abilities differ.

### 6.3.2 Impact of egg constituents on noodle properties

**Fresh noodles -** Adding egg fractions increased the extensibility at breakage but lowered the maximum force of Claire noodles (Table 6.1). These effects were more pronounced for egg white. In fresh Paragon noodles, only egg white addition significantly impacted Kieffer-rig parameters. The egg fractions had only little impact on the total work required for rupture of fresh noodles. The positive impact of egg (fraction) addition on the extensibility at breakage apparently outweighed its negative impact on the force measured during extension.

**Cooked noodles -** The use of whole egg in the salted noodle recipe had little impact neither on the optimal cooking time nor on water absorption and cooking loss. However, salted noodles made from recipes containing egg white or egg yolk had longer or shorter optimal cooking times respectively than did either whole egg or control noodles (Table 6.1). While egg white use increased and egg yolk use decreased water absorption and cooking loss, the impact of whole egg on cooking quality was intermediate to both.

Cooking increased the total work of whole egg and egg white noodles while it decreased that of egg yolk noodles. The use of whole egg significantly increased the extensibility at breakage, maximum force, and total work of cooked noodles. Egg white use increased the maximum force significantly but did not impact the extensibility of cooked noodles. It significantly increased total work for cooked Paragon noodles with egg white but not for cooked Claire noodles. In contrast, egg yolk addition had the complete opposite effect, *i.e.* it decreased the extensibility, the maximum force and total work of cooked noodles. It is remarkable that the impact of whole egg on Kieffer-rig parameters, in contrast to that on cooking quality, was not the combined effect of egg white and yolk. Noodles from the same wheat cv had equal protein content and flour to egg protein ratio, but different protein compositions and lipid contents. The next sections will investigate whether differences in noodle properties can be ascribed to differences in protein network formation.

### 6.3.3 Impact of egg constituents on protein network formation

#### 6.3.3.1 Importance of covalent cross-links

Protein in whole egg noodles polymerized faster and more protein was incorporated in the protein network than in control noodles (Figures 6.2.A and 6.2.E). Also, more protein was SS bound in whole egg than control noodles. Indeed, higher concentrations of DTT in the extraction medium were necessary to extract all protein (Figure 6.3). Protein in egg white noodles polymerized more rapidly (Figure 6.2) and to a larger extent, *i.e.* more protein was incorporated and interconnected by SS bonds (Figure 6.3), than in whole egg noodles. Less protein was incorporated (Figure 6.2) and lower

concentrations of DTT in SDS medium were required to extract protein from egg yolk noodles (Figure 6.3) than from whole egg and egg white noodles. Furthermore, protein in egg yolk noodles polymerized at a slower rate than protein in whole egg and egg white noodles but still faster than that in control noodles. In complex systems not all protein polymerizes at the same rate nor to the same extent. The extractability of egg protein in water from whole egg noodles and those of glutenin, α-, γ-, and ω-gliadin in DMSO medium were monitored as a function of heating time. Based on the kinetics of polymerization, calculated as in Section 6.2.7, it was concluded that in whole egg noodles, egg protein polymerized faster than gluten protein (Figure 6.4). After 6 min cooking, more egg protein remained extractable in water than gluten protein in DMSO medium. Glutenin polymerized faster than α- and γ-gliadin. Gliadin lacks free SH groups in contrast to glutenin. ω-Gliadin, which lacks both cysteine and cystine residues, remained fully extractable during cooking of whole egg noodles.

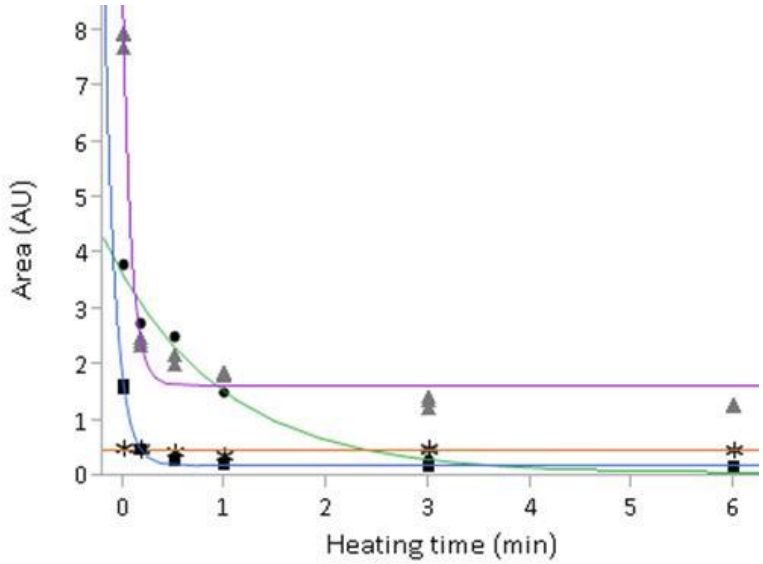


**Figure 6.4: Extractabilities (areas in SE-HPLC chromatograms) of hen egg proteins in water (Δ) and glutenin (□), ω-gliadin (∗), α- and γ-gliadin (○) in dimethyl sulfoxide:propanol:water (ratio 2:1:1, DMSO containing medium) of unsalted whole egg noodles made with flour of cultivar Paragon in water after heating for various times at 100 °C. Trend lines were fitted according to first-order kinetics. AU, arbitrary units.**

The rate and extent of protein polymerization in whole egg noodles corresponded to the values expected based on the data on protein polymerization in egg white and yolk noodles. The fast and extensive polymerization in egg white noodles reduced the flexibility of the protein network and resulted in low cooking quality as it impaired the capacity of the noodles to cope with starch swelling (Table 6.1). As noted for wheat globulin noodles, less protein in cooked egg yolk noodles was incorporated in the protein network and the extensibility was lower than that of cooked control noodles. The higher extent of cross-linking in cooked egg white and whole egg than in control

noodles increased the maximum force for both Claire and Paragon noodles (Table 6.1). The order of covalent protein network development (egg white > whole egg > egg yolk noodles) was positively related to cooking time, water absorption and cooking loss. These data support the view that high optimal cooking time is related to highly developed protein networks, which slow down water imbibition and therefore starch swelling and gelatinization. No clear differences in the latter were observed (Table 6.3).

**Table 6.3: Denaturation temperatures [onset, peak and conclusion temperature (T)] of noodle dough. Peak 1 and 2 contain mainly starch and starch-lipid complexes respectively. Hen egg proteins are present in both peaks.**

| Noodle type | Peak 1 | | | Peak 2 | | |
|---|---|---|---|---|---|---|
| | Onset T | Peak T | Conclusion T | Onset T | Peak T | Conclusion T |
| Control (C) | 51.2 ab | 60.0 b | 70.2 ab | 83.4 a | 93.5 ab | 100.5 a |
| (C) + Whole egg | 53.7 a | 60.4 ab | 70.9 ab | 76.7 b | 89.4 bc | 99.1 ab |
| (C) + Egg white | 52.3 ab | 60.8 a | 70.6 ab | 77.6 b | 89.7 abc | 100.6 a |
| (C) + Egg yolk | 51.2 b | 60.0 b | 69.8 ab | 76.3 b | 86.3 c | 95.8 b |
| (C) + Defatted egg yolk | 51.3 ab | 60.0 b | 69.5 b | 77.0 b | 89.0 bc | 97.9 ab |
| (C) + Defatted egg yolk + oil | 52.4 ab | 60.2 ab | 70.5 ab | 78.3 b | 89.0 bc | 98.2 ab |
| (C) + oil | 51.4 ab | 59.9 b | 71.4 a | 83.7 a | 94.0 a | 99.6 ab |

**Column values with the same letter are not significantly different (α = 0.05).**

#### 6.3.3.2 Importance of ionic interactions

Ionic interactions between egg and gluten proteins largely impact dough properties (Van Steertegem *et al.* 2013). They cannot be quantified, but it is possible to estimate attraction and repulsion forces between proteins in a system at a given pH. At pH 6.0, the pH of control and gluten dough (Table 6.1), gliadin [pH 5.8, calculated pI around 7.8 and in agreement with Wu and Dimler (1963)] has a net positive while glutenin (pH 8.3, calculated pI 5.8) a slightly net negative charge (Figure 6.5.A). Salt can shield the ionic interactions between gluten proteins. In fresh unsalted control and gluten noodles, ionic interactions between glutenin and gliadin resulted in more pronounced coherence of the partially developed gluten network and higher Kieffer-rig parameters than in their salted counterparts (Tables 6.1 and 6.2). Also, that the gluten network in wheat dough develops more slowly when salt is added (Van Steertegem *et al.* 2013) suggests a less continuous protein network in salted than in unsalted noodles. The latter can explain the shorter optimal cooking times for all salted noodle types (Tables 6.1 and 6.4).

**Table 6.4: Optimal cooking time, water absorption, cooking loss, protein content of cooking loss and protein extractabilities in sodium dodecyl sulfate medium (SDS-EP) of optimal cooked unsalted Paragon noodles made with different edible hen egg fractions and/or addition of olive oil. Extensibility until fracture, maximum force and total work of fresh and optimal cooked noodles.**

| Noodle type | Optimal cooking time (min) | Water absorption (g/g dm) | Cooking loss (% on dm) | Proteins in cooking loss (% on dm) | Extensibility (mm) | | Maximum force (N) | | Work (J) | | SDS-EP Cooked (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Fresh | Cooked | Fresh | Cooked | Fresh | Cooked | |
| Control (C) | 9 min | 2.11 b | 5.4 b | 9.0 d | 29.4 b* | 23.9 b | 1.19 b | 0.87 b | 23.0 a* | 11.5 b | 23.3 c |
| (C) + Whole egg | 9 min | 1.89 b | 4.9 b | 11.7 ab | 30.0 b* | 31.8 a | 1.27 b* | 1.56 a* | 22.3 a* | 27.8 a | 16.9 e |
| (C) + Egg white | 15 min | 3.37 a | 9.7 a | 12.9 a | 25.5 c* | 20.8 cd* | 1.04 c* | 0.84 b* | 16.7 bc* | 9.3 bc* | 14.0 f |
| (C) + Egg yolk | 4 min 30 sec | 1.25 c | 3.6 b | 12.6 a | 26.9 bc* | 19.4 de | 0.82 d* | 0.86 b | 12.6 d | 8.5 bc | 30.7 a |
| (C) + Defatted egg yolk | 11 min | 1.93 b | 5.8 b | 10.6 bc | 20.0 d | 15.1 f | 1.46 a | 0.95 b | 16.8 b | 7.4 c | 22.8 d |
| (C) + Defatted egg yolk + oil | 7 min | 1.09 c | 4.5 b | 11.7 abc | 27.3 bc | 16.6 ef | 0.74 d | 0.86 b | 11.2 d | 7.4 c | 22.9 c |
| (C) + oil | 8 min | 2.10 b | 5.0 b | 10.4 c | 40.8 a | 22.5 bc | 0.54 e | 0.60 c | 13.8 cd | 7.3 c | 24.7 b |

**Column values with the same letter are not significantly different ($\alpha = 0.05$).**

***Values are significantly different with the corresponding parameter of salted Paragon noodles within the same type (Table 6.2)**

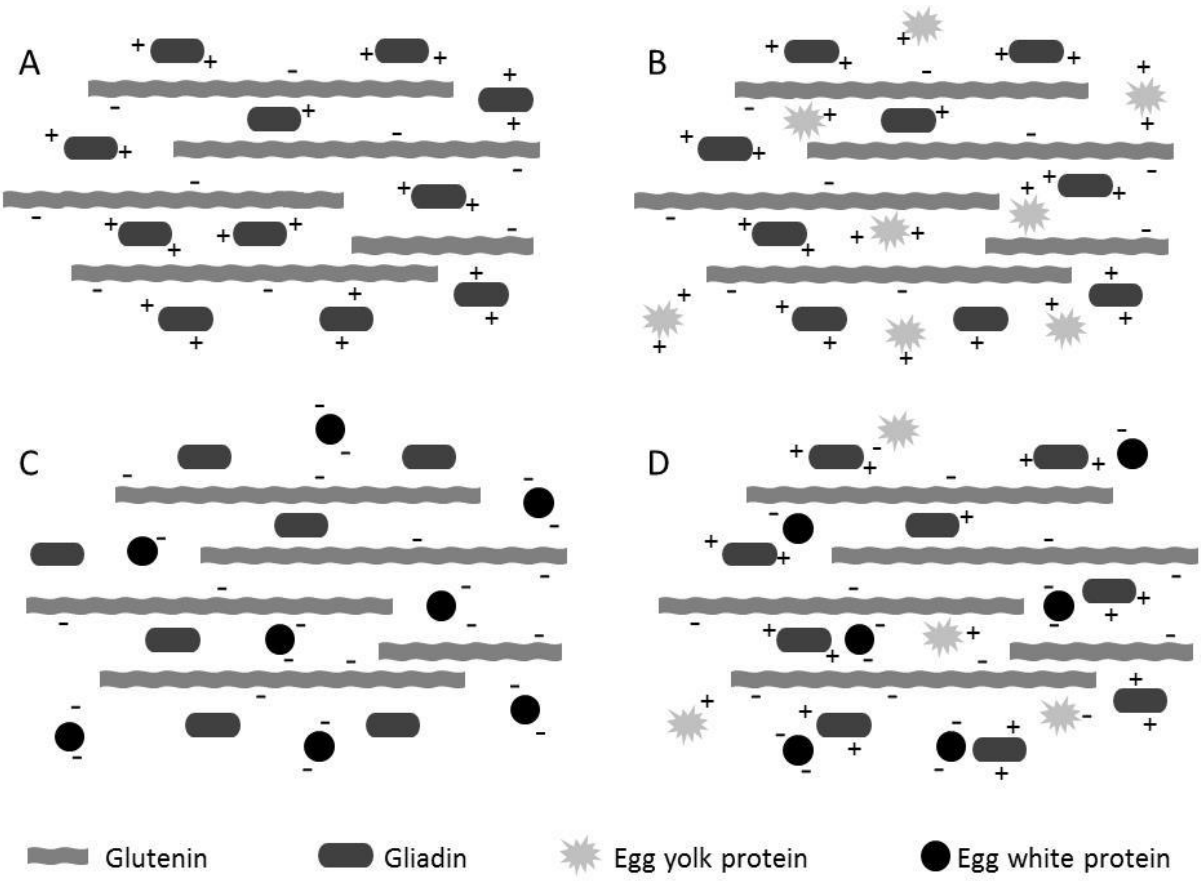


**Figure 6.5: Schematic visualization of ionic interactions in control (A), hen egg yolk (B), egg white (C) and whole egg noodles (D). Negative (-) and positive (+) surface charges are based on the average isoelectric point of protein mixtures and the actual dough pH.**

**Fresh noodles -** At the pH of egg yolk noodles (Table 6.1), the majority of egg yolk protein (Van Steertegem *et al.* 2013) and gliadin have a positive charge while glutenin has a slightly negative charge (Figure 6.5.B). Egg yolk protein can interact with glutenin the same way gliadin does, *i.e.* it increases dough viscosity and plasticity and reduces its elasticity (Shewry *et al.* 2001). The repulsion between egg yolk and gliadin proteins hinders their interaction. When salt was omitted from the recipe, the maximum force was lower and the extensibility higher (Tables 6.1 and 6.4). In egg white noodles (Table 6.1), glutenin and egg white protein are negatively charged while gliadin is hardly charged (Figure 6.5.C). Gliadin can still easily interact with glutenin. However, the repulsion between egg white protein and glutenin seem to hinder the development of a coherent protein network. All Kieffer-rig parameters were lower for unsalted fresh egg white than for control noodles (Table 6.4). Inclusion of salt in the recipe increased the extensibility of fresh egg white noodles but little resistance was measured during stretching (low maximum force and total work) (Table 6.1). The effect of salt predominated over the combined protein effect. Salt use probably resulted in a less continuous protein network and thereby lowered the total work needed for fracture. The majority of egg protein is negatively charged in whole egg noodle dough (Table 6.1), shielding the positively

charged gliadin (Figure 6.5.D). The ionic interactions between egg and gluten protein contributed to the coherence of the protein network noodles. Both in salted and unsalted Paragon noodles, no differences in total work were noted between fresh whole egg and control noodles. Salt addition had a larger impact on Kieffer-rig parameters than whole egg addition (Tables 6.1 and 6.4).

**Cooked noodles -** Salt addition had a different impact on the properties of cooked noodles than on those of fresh noodles. All Kieffer-rig parameters of unsalted cooked egg white and yolk noodles were similar but lower than those of whole egg noodles (Tables 6.1 and 6.4). While added salt did not impact the total work of whole egg and egg yolk noodles, the total work of egg white noodles drastically increased when salt had been used. As outlined above, protein networks developed faster in unsalted than in salted noodle dough. This combined with the fast cross-linking of egg white proteins led to a fixed protein network which could not cope with starch swelling during cooking. Finally, it is of note that salt also reduces protein polymerization during noodle cooking (Rombouts *et al.* 2014), thereby improving the flexibility of the protein network and the way it withstands starch swelling.

#### 6.3.3.3 Importance of hydrophobic interactions

Alamprese and others (2005b) suggested that hydrophobic interactions are relevant to egg pasta sheet extensibility. In their view, the hydrophobic patches of proteins in whole egg and egg yolk noodles can interact with egg yolk lipids and thereby reduce the frequency of the occurrence of hydrophobic interactions between proteins.

**Defatting egg yolk prior to noodle making -** The total work and maximum force of fresh defatted egg yolk noodles were higher and the extensibility lower than those of standard egg yolk noodles. The reduction of egg yolk lipids probably increased the level of hydrophobic protein-protein interactions and hence also the stiffness and strength of the protein network. Proteins in defatted egg yolk noodles polymerized faster during heating than that in egg yolk noodles (Figure 6.2). However, no difference in the level of protein incorporated in the protein network was noticed (Figure 6.3). Nevertheless, low levels of DTT (0.10%) in SDS medium released less protein from the protein network in defatted egg yolk noodles cooked for 12 min than from standard egg yolk noodles cooked for the same time (Figure 6.3). Thus, defatting egg yolk increased the level of protein-protein cross-links. The rate and extent of protein network formation in defatted egg yolk noodles were lower than those in egg white and whole egg noodles (Figures 6.2 and 6.3). We verified that the hexane treatment itself when tested on noodles containing (hexane-treated) BSA did not impact protein network formation of isolated proteins during noodle making (results not shown). Defatting egg yolk prior to using it in the noodle recipe increased the optimal cooking time and significantly decreased

the water absorption (Table 6.4). Also, less protein leached from noodles to the cooking water from defatted egg yolk noodles than from standard egg yolk noodles. Defatting significantly impacted neither the maximum force nor total work of optimally cooked egg yolk noodles.

**Addition of olive oil to noodle recipes -** To further investigate the impact of lipids on protein polymerization and noodle properties, olive oil was included in the recipes of control and defatted egg yolk noodles. The noodles with olive oil contained the same lipid level as standard egg yolk noodles. Olive oil in fresh control noodles caused a greater extensibility and reduced the maximum force and total work (Table 6.4). It seems to plasticize the noodle structure by obstructing protein-protein interactions. During cooking, the inclusion of oil in the recipe decreased the rate of protein polymerization significantly while no difference in the level of protein incorporated in the protein network was observed (Figures 6.2.A and 6.2.J). However, more protein was extractable with 0.10% DTT in SDS medium from oil containing than from control noodles (Figure 6.3). Thus, the use of oil slowed down protein network formation and decreased cross-linking between gluten proteins in noodles. As for control noodles, all protein of olive containing control noodles was extractable with 0.25% DTT in SDS medium. The optimal cooking time slightly decreased when the control noodles contained olive oil. No differences in cooking quality were noticed, except that more protein leached into the cooking water. While the use of olive oil did not impact the gelatinization temperature of starch (Table 6.3), the inferior protein network in the cooked noodles led to a lower maximum force and total work than for control noodles even if the noodle extensibility of cooked noodles was not influenced by the use of olive oil (Table 6.4).

The inclusion of olive oil in the recipe of fresh defatted egg yolk noodles increased the extensibility and decreased the maximum force and total work (Table 6.4). No significant differences were noted between extensibility parameters of standard egg yolk noodles and defatted egg yolk noodles containing olive oil. The rate of protein polymerization during cooking of defatted egg yolk noodles with oil was significantly lower than those for defatted egg yolk noodles and even standard egg yolk noodles (Figure 6.2). The extent of protein incorporation in the protein network in defatted egg yolk noodles containing olive oil was equal to that in defatted egg yolk noodles but lower than that in standard egg yolk noodles. More protein was extractable with 0.10% DTT in SDS medium from defatted egg yolk noodles with olive oil than from defatted egg yolk noodles (Figure 6.3). The level of protein extracted in 0.10% DTT in SDS medium of the former was similar as in standard egg yolk noodles. However, the protein network of defatted egg yolk noodles containing olive oil still was superior to that in egg yolk noodles (Figures 6.2 and 6.3).

Olive oil use decreased the optimal cooking time and water absorption more in defatted egg yolk noodles than in control noodles. In addition, it significantly impacted noodle maximum force and

total work when used in control noodles, but not in defatted egg yolk noodles (Table 6.4). The fatty acid composition of olive oil differs from that in egg yolk lipids (Belitz *et al.* 2009). Because olive oil impacts protein network formation and properties of defatted egg yolk noodles differently than those of control noodles, it is hypothesized that rather than only exerting physical hindrance, lipids interact with egg yolk lipoprotein and hinder their subsequent polymerization.

#### 6.3.3.4 Importance of hydrogen bonds

The role of hydrogen bonds in gluten networks is undisputed (Belton 1999). Dough constituents interact with water. This reduces its mobility. Depending on the degree of interaction, different proton populations can be distinguished (Bosmans *et al.* 2012). Based on the model systems described by Bosmans and others (2012) and Luyts and others (2013) the proton populations in fresh and optimally cooked noodles (Figure 6.6) could be assigned. Proton population A, with lowest mobility (lowest $T_2$), is not in contact with water and contains rigid non-exchanging CH protons of starch, gluten and egg protein. This population decreases during cooking as a result of starch gelatinization (Bosmans *et al.* 2012; Luyts *et al.* 2013). The remainders are rigid CH protons in the protein network and, in cooked and cooled noodles, crystalline amylose. Proton populations B and C are similar and consist of CH protons in amorphous starch regions and protein aggregates in little contact with water. Population D contains exchangeable water protons within and directly surrounding starch granules and protein aggregates. The relative fraction of proton populations B, C and D decreased during cooking as noodle constituents increasingly came into contact with water (Figure 6.6, Table 6.5). Finally, protons in population E had the highest mobility and contained lipid protons in fresh noodles. The absorbed cooking water was responsible for the increase in proton population E during cooking. While bulk water has a $T_2$ of about 2.5-3.0 sec (Luyts *et al.* 2013), the absorbed cooking water was bound in the noodle structure with $T_2$ around 60 msec.

**Table 6.5: Spin-spin relaxation times ($T_2$) and corresponding peak areas for different proton populations present in fresh and optimally cooked unsalted Paragon noodles made with different edible wheat and hen egg fractions. AU, arbitrary units. Standard deviations are between brackets.**

| Noodle type | | Population A | | Population C | | Population D | | Population E | |
|---|---|---|---|---|---|---|---|---|---|
| | | $T_2$ (µsec) | Area (AU) | $T_2$ (msec) | Area (AU) | $T_2$ (msec) | Area (AU) | $T_2$ (msec) | Area (AU) |
| Control (C) | Fresh | 13 (0) | 19623 (204) | 0.45 (0.02) | 909 (5) | 4.32 (0.06) | 6703 (19) | 60.00 (0.00) | 61 (4) |
| | Cooked | 14 (0) | 3701 (392) | 0.50 (0.10) | 813 (91) | 4.87 (0.51) | 1173 (94) | 56.53 (4.37) | 14403 (209) |
| (C) + Gluten | Fresh | 13 (0) | 18620 (244) | 0.61 (0.05) | 1377 (181) | 5.00 (0.36) | 6478 (100) | 63.33 (11.55) | 79 (10) |
| | Cooked | 13 (1) | 3586 (150) | 0.57 (0.06) | 694 (73) | 6.30 (1.04) | 991 (45) | 71.37 (5.69) | 14883 (298) |
| (C) + Whole egg | Fresh | 13 (0) | 18476 (447) | 0.60 (0.05) | 1147 (160) | 3.52 (1.04) | 6932 (605) | 76.67 (4.62) | 452 (14) |
| | Cooked | 13 (1) | 4023 (143) | 0.53 (0.15) | 696 (37) | 4.73 (0.75) | 1154 (115) | 43.20 (1.57) | 14111 (174) |
| (C) + Egg white | Fresh | 13 (0) | 20103 (124) | | | 1.64 (0.09) | 7453 (104) | 53.33 (5.77) | 67 (5) |
| | Cooked | 13 (1) | 3514 (199) | 0.40 (0.00) | 355 (28) | 6.33 (1.53) | 875 (93) | 66.83 (3.72) | 15844 (99) |
| (C) + Egg yolk | Fresh | 13 (0) | 16995 (276) | 0.49 (0.01) | 866 (33) | 4.32 (0.05) | 6912 (104) | 87.33 (0.58) | 1077 (29) |
| | Cooked | 13 (1) | 4331 (308) | 0.49 (0.09) | 978 (40) | 4.50 (0.26) | 1495 (114) | 43.10 (0.98) | 13205 (169) |
| (C) + Defatted egg yolk | Fresh | 13 (0) | 18914 (49) | 0.44 (0.02) | 1013 (63) | 4.11 (0.08) | 6682 (39) | 56.67 (15.28) | 54 (13) |
| | Cooked | 13 (1) | 3547 (80) | 0.49 (0.02) | 910 (60) | 4.40 (0.17) | 1447 (63) | 50.13 (2.73) | 11995 (973) |

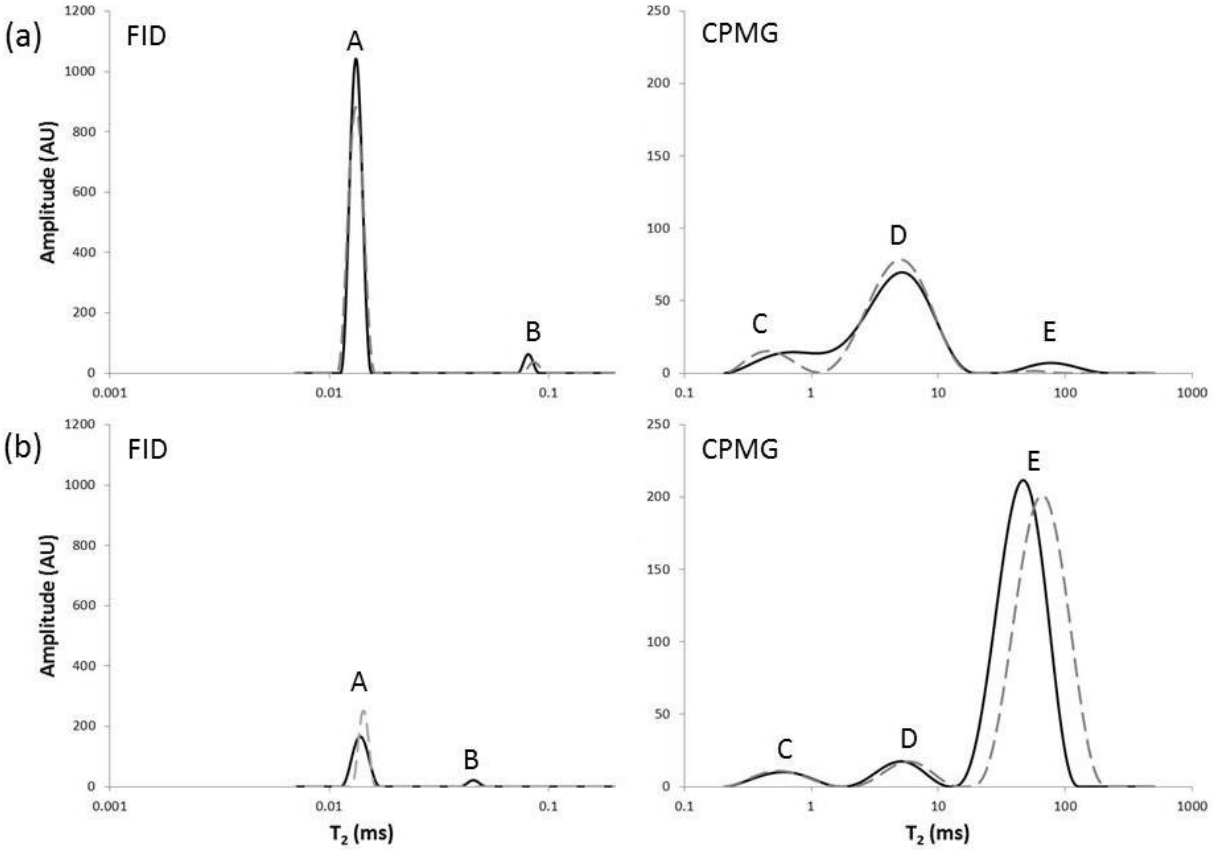


**Figure 6.6: Free induction decay (FID) and Carr-Purcell-Meiboom-Gill (CPMG) spectra of fresh (a) and optimally cooked (b) control (---) and whole egg (-) noodles. The different proton populations are indicated with capital letters in order of increasing mobility. AU, arbitrary units.**

**Fresh noodles -** The main differences in NMR spectra of fresh control and gluten noodles were noted for populations C and D. In fresh gluten noodles, more protons were in little contact with water (population C) and less protons were exchanging with those of water (population D) than in control noodles (Table 6.5). However, the average mobility of both populations was higher in fresh gluten than in control noodles. Thus, a higher degree of hydrogen bond exchange between noodle constituents occurs as a result of the use of extra gluten. Whether this contributes to the improved dough properties upon wheat gluten addition remains unclear at present. Also in fresh whole egg noodles population C had higher mobility than in control noodles (Figure 6.6, Table 6.5). No distinction between populations C and D could be made in fresh egg white noodles. Water protons were less mobile, thus more strongly bound, in fresh egg white noodles than in all other noodle types. Also, defatting decreased the proton mobility and the fraction of protons occurring in population D in egg yolk noodles. Proton population E, which contained lipid protons, was highest and most mobile for fresh egg yolk noodles, and lowest for control, gluten, egg white and defatted egg yolk noodles.

**Cooked noodles –** The moisture gradient during cooking of noodles homogenizes after cooking (Kojima *et al.* 2001). During cooking, noodles absorb water, starch swells and gelatinizes, and proteins extend their covalent network, resulting in decreases in proton populations A, B, C and D, and increases in proton population E for all noodles (Figure 6.6, Table 6.5). Differences in cooking time led to differences in water absorption and levels of protons in population E. However, it should be noted that the rate of water diffusion in wheat noodles decreases over cooking time (Maeda *et al.* 2009). Also, the mobility of population E protons generally seemed to increase with cooking time except for whole egg noodles (Tables 6.4 and 6.5). Such noodles, which had the same optimal cooking time as control noodles (Table 6.4), had lower $T_2$ values for population E after cooking (Table 6.5, Figure 6.6). In noodles cooked for 20 min, and thus beyond the optimum cooking time, the $T_2$ values of this population were similar for all noodle types except for whole egg noodles, in which water protons were more homogeneously bound and thus had a lower average $T_2$ value (results not shown). As demonstrated above, as starch swells and the noodle volume increases, ionic and hydrophobic protein-protein interactions had little impact on total work in Kieffer-rig extensibility testing (Table 6.4). The NMR data indicate that hydrogen bonds between flour, egg constituents and water together with the formed covalent network in noodles can determine the coherence and flexibility of the noodle structure.

## 6.4 Conclusion

This Chapter demonstrated that both non-covalent (ionic, hydrophobic and hydrogen) interactions and cross-linking reactions between proteins impact extensibility parameters and cooking characteristics of noodles. During cooking, starch swells. This reduces the importance of ionic and hydrophobic interactions on noodle extensibility. Cooking quality and properties of cooked noodles are largely determined by the balance between covalent protein network formation and starch gelatinization. The covalent protein network of whole egg containing noodles was inferior to that of only egg white containing noodles but superior to that of only egg yolk containing noodles. However, the rapidly formed protein network in egg white noodles could not cope with starch swelling. These noodles had high optimal cooking times, low cooking quality and low total work during Kieffer-rig extensibility tests. In contrast, the covalent protein network in egg yolk containing noodles was only moderately developed. This resulted in low optimal cooking times and noodle strength. It is suggested that not only the timing and extent of covalent network formation but also hydrogen interactions are the main determinants of extensibility of cooked noodles.

# Chapter 7

# The impact of various globular proteins on protein network formation in noodles

## 7.1 Introduction

Both protein quantity and quality impact protein network formation in egg noodles as demonstrated in Chapter 6. Both covalent and non-covalent network formation impact the properties (optimal cooking time, cooking quality and Kieffer-rig extensibility) of fresh and cooked egg noodles. However, the impact of protein characteristics on protein network formation and properties of noodles remains to be elucidated. As explained in Chapter 5, mixtures of gluten with globular proteins containing high levels of accessible free SH groups and hydrophobic patches upon heating at 100 °C enhance each other's polymerization.

To study the relation between protein characteristics, protein network formation and noodle properties, different well characterized food proteins were included in noodle recipes. Higher levels of accessible free SH groups and hydrophobic patches are expected to increase gluten polymerization in noodles and thereby enhance noodle properties. To that end, covalent network formation was evaluated based on the loss of extractability in SDS medium. The influence of ionic, hydrophobic or

hydrogen interactions between proteins on noodle properties was studied by comparing noodles with and without salt, olive oil or urea addition respectively.

## 7.2 Materials and methods

### 7.2.1 Materials and characterization thereof

To link protein characteristics with protein network formation in noodles and product properties, the isolated proteins used and characterized in Chapter 5 were used to replace whole egg in the egg noodle recipe described in Chapter 6.

**Wheat flour** cv Paragon (13.9% protein on dm) and **soy glycinin** (100.0% protein on dm) were obtained as in Section 3.2.1. **Hen egg lysozyme** (from chicken egg white, 100.0% protein on dm) and **ovalbumin** (albumin chicken egg grade III, 94.1% protein on dm) were from Sigma-Aldrich. Ovalbumin (6.67 mg/ml) was shaken for 24 h at 55 °C in 0.10 M glycine-sodium hydroxide buffer (pH 9.9) to convert it into **S-ovalbumin**. After dialysis for 24 h against water, S-ovalbumin (91.6% protein on dm) was freeze-dried and ground. **BSA** (fraction V for biochemistry, 98.2% protein on dm) was from Acros Organics. Commercial olive oil (Pietro Coricelli, extra virgin) was used. All chemicals were of analytical grade and from Sigma-Aldrich unless specified otherwise. DTT, disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International. Moisture and protein content were determined as in Section 6.2.1 and Section 3.2.2, respectively.

### 7.2.2 Noodle production and cooking

Noodles with a constant ratio of flour to model proteins of 2:1, moisture and protein contents of respectively 33.9% and 18.7% on dm were made from recipes containing only wheat flour, model proteins (BSA, ovalbumin, S-ovalbumin, soy glycinin and lysozyme) and deionized water as described in Section 6.2.2 in triplicate. Control noodles without model proteins had a protein content of 14.2% on dm. Salt, urea or olive oil (each 3.0% on dm) were added to control noodle recipes and those of noodles containing BSA, ovalbumin or lysozyme. These noodle types were made in duplicate batches. Fresh noodle strands (20.0 g) were cooked in 500 ml deionized water to optimum as well as for 30 sec, 1, 3, 6, 12 and 20 min, recovered and immediately cooled in 200 ml deionized water at 23 °C as in Section 6.2.2.

### 7.2.3 Noodle properties

Water absorption, cooking loss, protein in cooking loss, dough pH and Kieffer-rig extensibility parameters were obtained as in Section 6.2.3.

### 7.2.4 Low resolution proton nuclear magnetic resonance

Proton mobility distributions in fresh and cooked noodles were determined as in Section 6.2.5. Five different proton populations were distinguished using $^{1}$H-NMR and assigned to noodle constituents based on starch-water, gluten-water, flour-water (Bosmans *et al.* 2012) and egg-water model systems (Luyts *et al.* 2013).

### 7.2.5 Size exclusion high performance chromatography

The protein extractable in SDS medium and its MW distribution were determined in triplicate as optimized in Chapter 3. To evaluate extractability under reducing conditions, 0.10%, 0.25%, 0.50% or 1.00% (w/v) DTT was included in the SDS medium as in Section 6.2.6.

### 7.2.6 Kinetics of extractability loss in sodium dodecyl sulfate containing medium of cooked noodles

The kinetics of protein polymerization were studied as in Section 6.2.7.

### 7.2.7 Statistical analysis

Significant differences ($\alpha = 0.05$) based on at least three measurements, were determined with one-way ANOVA. Trend lines, corresponding equations and goodness of fit ($R^2$) of protein polymerization kinetics were estimated as in Section 6.2.8.

## 7.3 Results

### 7.3.1 Impact of different food proteins on noodle properties

**Fresh noodles –** The addition of BSA and soy glycinin had only little impact on the total work required to rupture fresh noodles (Table 7.1). The positive impacts of BSA and soy glycinin on the force during extension apparently outweighed their negative impact on the extensibility at breakage. While inclusion of lysozyme did not impact any of the Kieffer-rig extensibility parameters, that of (S-)ovalbumin resulted in fresh noodles with lower extensibility and total work at breakage than noted for control noodles.

**Optimally cooked noodles –** Addition of BSA and soy glycinin had limited impact on optimal cooking time, water absorption and cooking loss (Table 7.2). Nevertheless, the maximum force during extension and total work needed for fracturing was higher for noodles containing BSA and soy glycinin than for control noodles (Table 7.1). Addition of BSA or soy glycinin did not significantly impact the extensibility readings. Inclusion of (S-)ovalbumin in the noodle recipe increased the

optimal cooking time, decreased the extensibility but had no significant impact on total work of optimally cooked noodles (Tables 7.1 and 7.2). Water absorption and cooking losses were higher for S-ovalbumin containing than for control noodles (Table 7.2). Also, more protein leached out from S-ovalbumin containing noodles into the cooking water than from control noodles. Finally, noodles from lysozyme containing recipes had the lowest optimal cooking time (Table 7.2). Their cooking quality was similar to that of control noodles except that more protein leached into the cooking water and that they required higher maximum force and total work (Table 7.1).

**Overcooked noodles –** The water absorption after 20 min of cooking was similar for all noodle types (Table 7.2). Only noodles containing lysozyme had higher cooking losses with higher protein levels therein than did control noodles. Cooking beyond the optimum consistently resulted in significant decreases in all Kieffer-rig extensibility parameters, with the exception that the extensibility loss of glycinin containing noodles during overcooking was not significant (Table 7.1). Noodles containing (S-)ovalbumin had lower extensibility at breakage than the other noodle types. Only addition of BSA or soy glycinin significantly increased the total work in overcooked noodles.

**Table 7.1: Extensibility at breakage, maximum force during extension and total work needed for fracture of fresh, optimally cooked and overcooked (20 min) noodles made with different model proteins [bovine serum albumin (BSA), soy glycinin, hen egg (S-)ovalbumin and lysozyme].**

| Noodle type | Fresh noodles | | | Optimally cooked noodles | | | Overcooked noodles | | |
|---|---|---|---|---|---|---|---|---|---|
| | Extensibility (mm) | Maximum force (N) | Work (J) | Extensibility (mm) | Maximum force (N) | Work (J) | Extensibility (mm) | Maximum force (N) | Work (J) |
| Control (C) | 29.4 a | 1.19 c | 23.0 a | 23.9 a | 0.87 c | 11.5 cd | 21.3 a | 0.66 c | 7.9 bc |
| (C) + BSA | 23.0 b | 1.48 b | 21.1 a | 24.9 a | 1.28 b | 17.3 b | 20.7 a | 1.05 a | 11.7 a |
| (C) + Glycinin | 21.0 b | 1.81 a | 21.9 a | 24.3 a | 1.30 b | 16.8 b | 22.7 a | 0.96 a | 12.2 a |
| (C) + Ovalbumin | 20.2 b | 0.83 d | 11.0 b | 19.2 b | 0.98 c | 9.9 c | 14.5 b | 0.75 bc | 5.6 c |
| (C) + S-Ovalbumin | 21.2 b | 1.24 c | 14.6 b | 20.2 b | 1.31 b | 14.1 bc | 17.0 b | 1.04 a | 9.8 ab |
| (C) + Lysozyme | 28.3 a | 1.27 c | 24.4 a | 25.8 a | 1.57 a | 23.0 a | 21.6 a | 0.95 ab | 11.3 ab |

**Values in the same column with the same letter are not significantly different (α = 0.05).**

**Table 7.2: Dough pH, optimal cooking time, water absorption, cooking loss, protein content of cooking loss for optimally cooked and overcooked (20 min) noodles. Noodles were made with different model proteins [bovine serum albumin (BSA), soy glycinin, hen egg (S-)ovalbumin and lysozyme] in the recipe. Dry matter (dm).**

| Noodle type | Fresh noodles | Optimally cooked noodles | | | | Overcooked noodles | | |
|---|---|---|---|---|---|---|---|---|
| | pH | Optimal cooking time | Water absorption (g/g dm) | Cooking loss (% on dm) | Proteins in cooking loss (% on dm) | Water absorption (g/g dm) | Cooking loss (% on dm) | Proteins in cooking loss (% on dm) |
| Control (C) | 5.9 c | 9 min | 2.11 bc | 5.4 b | 10.5 c | 3.43 a | 7.5 b | 8.9 bc |
| (C) + BSA | 6.1 b | 9 min | 2.26 bc | 6.3 b | 9.0 c | 3.09 a | 7.8 ab | 8.2 c |
| (C) + Glycinin | 5.9 c | 8 min | 1.96 c | 5.4 b | 10.9 bc | 3.26 a | 7.7 b | 9.9 bc |
| (C) + Ovalbumin | 6.2 ab | 13 min | 2.78 ab | 6.7 b | 10.2 c | 3.41 a | 7. 8 ab | 9.6 bc |
| (C) + S-Ovalbumin | 6.4 a | 13 min 30 sec | 3.40 a | 10.8 a | 14.9 b | 3.23 a | 10.3 ab | 13.6 b |
| (C) + Lysozyme | 5.3 d | 4 min 30 sec | 1.65 c | 5.4 b | 30.1 a | 2.66 a | 10.4 a | 27.1 a |

**Values in the same column with the same letter are not significantly different (α = 0.05).**

## 7.3.2 Protein network formation in noodles with different proteins

### 7.3.2.1 Non-covalent network formation

The contribution of ionic or hydrophobic interactions or hydrogen bonds to protein network formation in noodles made with different model proteins was studied by adding salt, olive oil or urea respectively (Table 7.3).

**Impact of salt addition** – Inclusion of salt in the recipe significantly decreased the total work for fresh control, BSA or lysozyme containing noodles but had no impact on total work of fresh noodles made with ovalbumin addition [Table 7.3, symbols (>, <, =) illustrate significant differences between Kieffer-rig extensibility results without or with salt, urea or olive oil addition for the corresponding noodle types]. The positive impact of salt addition on the extensibility of ovalbumin containing noodles outweighed its negative impact on the force measured during extension. Even after 20 min of cooking, the negative impact of salt on the Kieffer-rig extensibility parameters of noodles containing BSA or lysozyme remained. In addition, salt use largely increased water absorption in these noodle types. In contrast, salt impacted neither the Kieffer-rig extensibility parameters nor the water absorption of control and ovalbumin overcooked noodles.

**Impact of olive oil addition** – In fresh noodles, the use of olive oil resulted in a lower maximum force and total work for control noodles and noodles from BSA or lysozyme containing recipes (Table 7.3, symbols). However, it did not impact the total work recorded for fresh ovalbumin containing noodles. Here, the positive impact of olive oil on extensibility outweighed its negative impact on the force measured during extension. Surprisingly, cooking of noodles containing both olive oil and ovalbumin or BSA for 20 min did not alter the total work needed to fracture noted for their fresh counterparts. Olive oil addition decreased the Kieffer-rig extensibility parameters of overcooked lysozyme containing noodles while those of control and BSA noodles remained the same. Overcooked ovalbumin and olive oil containing noodles even required more work to fracture than their counterparts not containing olive oil.

**Table 7.3: Extensibility at breakage, maximum force during extension, total work needed for fracture, water absorption and cooking loss of fresh and overcooked (20 min) noodles made with different model proteins [bovine serum albumin (BSA), hen egg ovalbumin and lysozyme] and salt, olive oil or urea as additive.**

| Additive | Noodle type | Fresh noodles | | | Overcooked noodles | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Extensibility (mm) | Maximum force (N) | Work (J) | Extensibility (mm) | Maximum force (N) | Work (J) | Water absorption (g/ g dm) | Cooking loss (% on dm) |
| Salt | Control (C) | 23.5 a < | 1.00 a < | 13.9 a < | 21.1 a = | 0.74 a = | 8.5 a = | 3.29 (0.03) | 10.1 (0.5) |
| | (C) + BSA | 19.0 b = | 0.59 c < | 5.6 c < | 16.6 b < | 0.75 a < | 6.6 ab < | 3.88 (0.30) | 12.0 (0.5) |
| | (C) + Ovalbumin | 26.5 a > | 0.67 c < | 10.3 b = | 14.9 b = | 0.76 a = | 6.2 b = | 3.46 (0.28) | 10.3 (0.6) |
| | (C) + Lysozyme | 12.9 c < | 0.84 b < | 6.2 c < | 16.3 b < | 0.58 b < | 5.2 b < | 3.99 (0.20) | 13.0 (0.4) |
| Olive oil | Control (C) | 25.9 a < | 1.04 b < | 16.2 a < | 20.9 a = | 0.73 b = | 8.5 b = | 3.42 (0.25) | 6.5 (0.3) |
| | (C) + BSA | 18.1 b = | 1.22 a < | 12.9 b < | 20.9 a = | 1.09 a = | 12.0 a = | 3.62 (0.01) | 6.9 (0.2) |
| | (C) + Ovalbumin | 25.5 a > | 0.64 c < | 8.9 c = | 17.9 b > | 1.07 a > | 10.4 ab > | 3.32 (0.32) | 6.7 (0.1) |
| | (C) + Lysozyme | 26.1 a = | 0.67 c < | 11.4 b < | 16.9 b < | 0.57 c < | 5.0 c < | 3.71 (0.03) | 8.2 (1.9) |
| Urea | Control (C) | 22.5 a < | 0.73 a < | 10.2 a < | 18.5 a < | 0.63 b = | 6.0 ab < | 3.50 (0.05) | 10.0 (1.2) |
| | (C) + BSA | 17.3 b < | 0.77 a < | 8.2 b < | 17.5 ab < | 0.84 a < | 7.9 a < | 3.14 (0.08) | 11.8 (0.1) |
| | (C) + Ovalbumin | 23.5 a = | 0.57 b < | 8.4 ab < | 15.2 b = | 0.88 a = | 7.6 a = | 3.06 (0.15) | 10.0 (0.2) |
| | (C) + Lysozyme | 25.4 a = | 0.60 b < | 9.9 ab < | 16.5 ab < | 0.61 b < | 5.4 b < | 3.72 (0.10) | 11.1 (0.6) |

**Values in the same column with the same additive and letter are not significantly different (α = 0.05). Values with < or > are significantly lower or higher, respectively than the corresponding value in Table 7.1 without additive. Values with = are not significantly different from the corresponding value without additive in Table 7.1. Standard deviations are between brackets.**

**Impact of urea addition** – Addition of urea decreased the maximum force and total work for all fresh noodle types and at the same time decreased differences between the different noodle types (Tables 7.3, symbols and letters). The decrease in total work was most pronounced in fresh BSA containing noodles and less so in fresh ovalbumin containing noodles. In addition, when the impact of hydrogen bonds in control, BSA, ovalbumin and lysozyme containing overcooked noodles was studied with $^{1}$H-NMR, similar proton mobility distributions were obtained for the first three noodle types (results not shown). However, the transverse relaxation times ($T_2$) of the proton population with highest mobility (population E) which is ascribed to absorbed cooking water, was significantly lower for noodles containing lysozyme ($T_2$ 58.20 msec ± 5.09 msec) than for control noodles ($T_2$ 92.36 msec ± 6.43 msec). Also in optimally cooked noodles, protons were more tightly bound when lysozyme was part of the recipe (results not shown). Protons corresponding to population E were more mobile in ovalbumin containing noodles ($T_2$ 68.67 msec ± 0.91 msec) than in control noodles ($T_2$ 56.53 msec ± 4.37 msec).

In summary, inclusion of urea in the recipe of fresh control noodles decreased all Kieffer-rig parameters to a larger extent than did inclusion of salt or olive oil. Addition of both urea and model proteins to fresh noodles recipes had no or a slightly negative impact on Kieffer-rig extensibility parameters. For fresh BSA and lysozyme containing noodles, addition of salt caused the largest decrease in total work. The total work of fresh ovalbumin containing noodles was lower when urea was part of the recipe. Addition of model proteins to noodle recipes containing salt, olive oil or urea mostly had a negative impact on the Kieffer-rig extensibility parameters of fresh noodles (Table 7.3, letters). Addition of salt, olive oil or urea had a greater impact on Kieffer-rig extensibility parameters of fresh noodles than addition of proteins, except for what was observed with (S-)ovalbumin. Only urea caused a decrease in the extensibility and total work of control noodles cooked for 20 min (Table 7.3, symbols). While salt or urea decreased all Kieffer-rig extensibility parameters of BSA and lysozyme containing overcooked noodles, they did not impact these parameters for ovalbumin containing noodles. BSA and ovalbumin containing overcooked noodles had the highest Kieffer-rig extensibility values when using olive oil. Olive oil also decreased all Kieffer-rig extensibility parameters for lysozyme containing overcooked noodles

#### 7.3.2.2 Covalent network formation

Protein in noodles containing **BSA** polymerized faster (high k value) during cooking and more protein was incorporated in the protein network (lower $[y]_{minimal}$ value) than noted for control noodles (Figures 7.1.A and 7.1.B). Also, higher concentrations of DTT in the SDS medium were necessary to extract all protein from noodles containing BSA heated in boiling water for 12 min at 100 °C than

from control noodles (Figure 7.2). Thus, proteins interconnected more through SS bonds during cooking in noodles containing BSA than in control noodles.

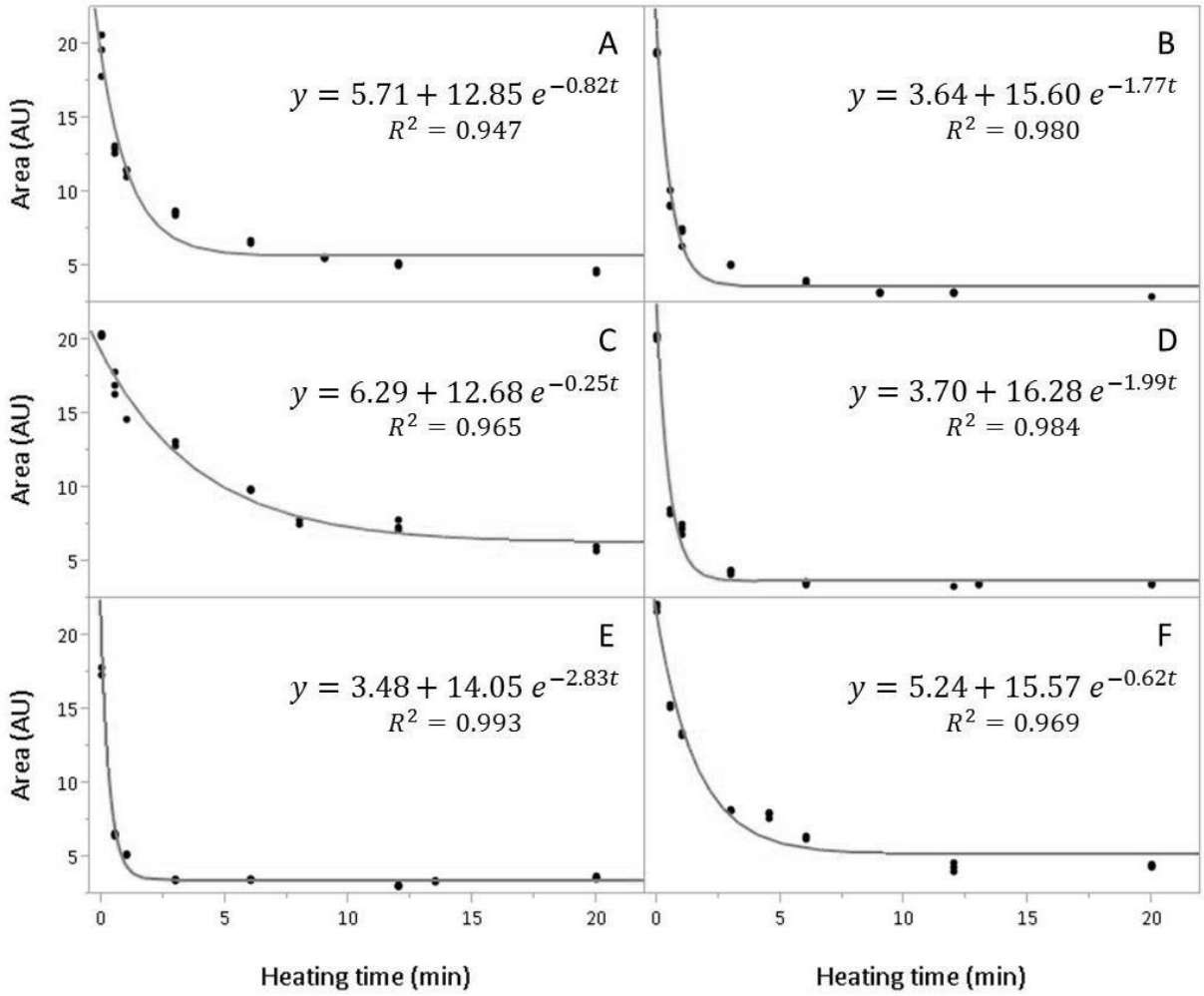


**Figure 7.1: Extractabilities (areas in SE-HPLC chromatograms) of proteins in sodium dodecyl sulfate (SDS) medium after heating at 100 °C of noodles made with flour (control, A) or from recipes including bovine serum albumin (BSA, B), soy glycinin (C), hen egg ovalbumin (D), S-ovalbumin (E) and lysozyme (F). Trend lines and their corresponding formula, expressed as Equation 5.3, and goodness of fit ($R^2$) were achieved applying first-order kinetics. AU, arbitrary units.**

Inclusion of **soy glycinin** in the noodle recipe decreased the rate of polymerization during cooking (Figures 7.1.A and 7.1.C). More protein was extractable in SDS and 0.10% DTT containing medium from 12 min cooked noodles containing soy glycinin than from control noodles (Figure 7.2). Less protein was incorporated and interconnected in soy glycinin containing than in control noodles (Figures 7.1.A, 7.1.B and 7.2). During cooking, the level of glutenin (eluting between *ca.* 5 min and 8 min) and α- and γ-gliadins (eluting between *ca.* 9 min and 9 min 30 sec) extractable in SDS medium decreased faster in BSA than in soy glycinin containing noodles (Figures 7.3.A and 7.3.B).

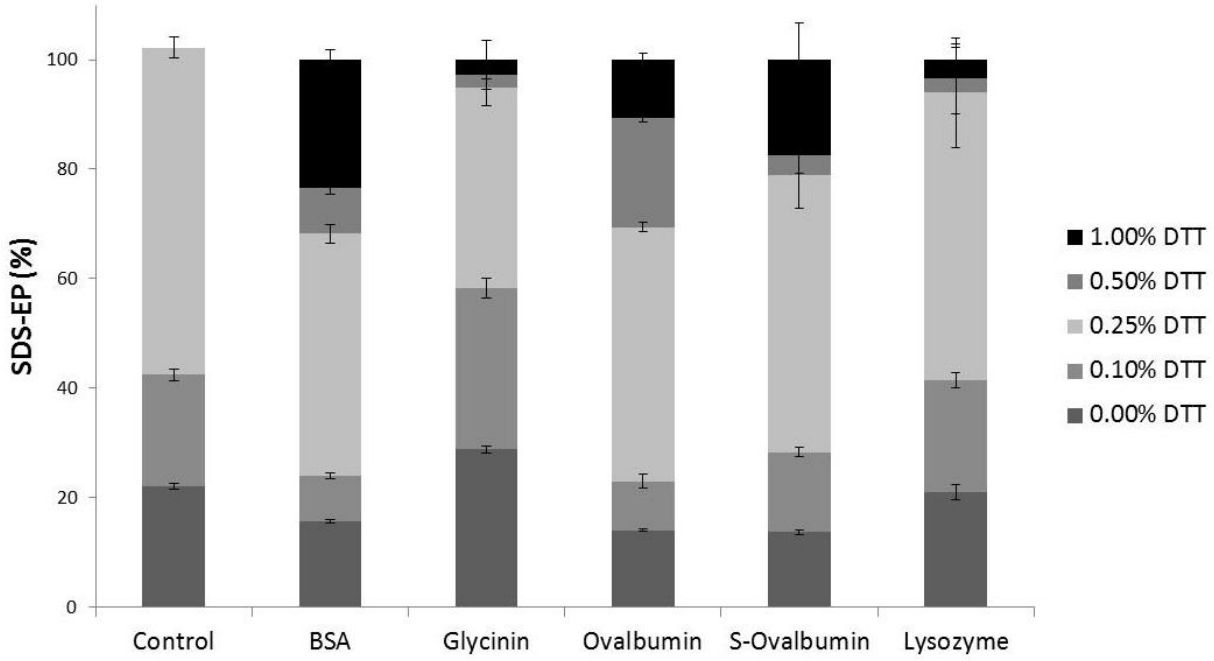


**Figure 7.2: Extractability of proteins in sodium dodecyl sulfate medium (SDS-EP) with increasing dithiothreitol (DTT) concentrations for cooked noodles (12 min at 100 °C) from flour only (control) or from flour and bovine serum albumin (BSA), soy glycinin, hen egg ovalbumin, S-ovalbumin or lysozyme, expressed as a percentage on the corresponding sample in SDS/DTT medium. Standard deviations are given with error bars.**

Addition of **ovalbumin** increased the polymerization rate of protein in noodles even more than did that of BSA (Figures 7.1.B and 7.1.D). More protein was incorporated and interconnected during cooking in ovalbumin containing than in control noodles (Figures 7.1.A, 7.1.D and 7.2). Protein polymerized faster but to the same extent as in noodles containing BSA (Figures 7.1.B and 7.1.D). As in BSA containing noodles, glutenin and α- and γ-gliadins were rapidly incorporated in the protein network of ovalbumin containing noodles (Figures 7.3.A and 7.3.C). After 3 min of cooking, more α- and γ-gliadins had become unextractable in SDS medium in noodles containing ovalbumin than in those containing BSA. However, more protein was extracted with 0.50% DTT in this medium from ovalbumin than from BSA containing noodles (Figure 7.2). Thus, the protein network of BSA containing noodles was better developed than that of ovalbumin containing noodles.

During production of **S-ovalbumin**, a small portion of the protein cross-linked already through SS bonds (Section 5.3.1). Protein polymerized faster but to the same extent during cooking in noodles containing S-ovalbumin than when they contained ovalbumin (Figures 7.1.D and 7.1.E). After 12 min of cooking, more protein was extractable with low concentrations of DTT (0.10% and 0.25%) from noodles containing S-ovalbumin than from those containing ovalbumin (Figure 7.2). However, a protein fraction in noodles containing S-ovalbumin was bound more strongly in the structure as it required higher DTT concentrations to become extractable.

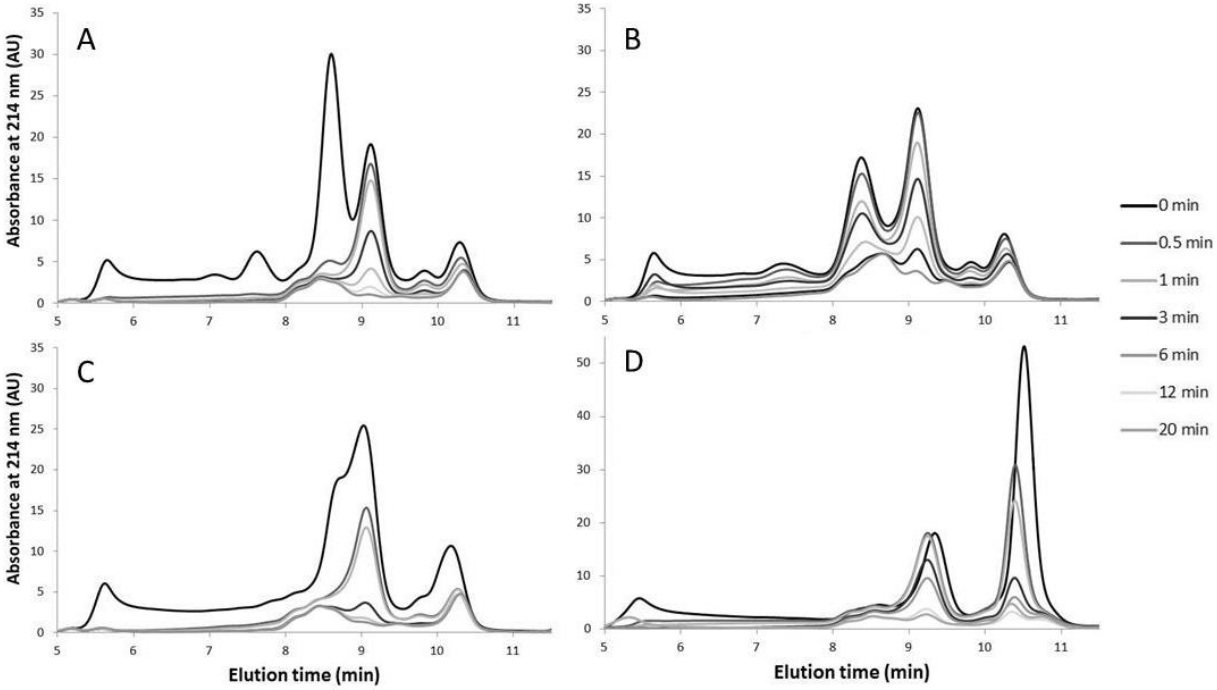


**Figure 7.3: SE-HPLC profile of protein extracts in sodium dodecyl sulfate (SDS) medium of noodles made from flour and bovine serum albumin (BSA, A), soy glycinin (B), ovalbumin (C) and lysozyme (D) after heat treatment at 100 °C for various times in water. AU, arbitrary units.**

Addition of **lysozyme** slowed down protein polymerization in noodles during cooking (Figures 7.1.A and 7.1.F). The decrease in polymerization rate by lysozyme addition was less pronounced than that when using soy glycinin (Figures 7.1.C and 7.1.F). Even though lysozyme does not contain free SH groups, it became rapidly incorporated in the protein network during cooking (Figure 7.3.D, elution between *ca.* 10 min and 11 min 10 sec). In the end, protein polymerized and interconnected to the same extent in lysozyme containing and control noodles (Figures 7.1.A, 7.1.F and 7.2).

# 7.4 Discussion

## 7.4.1 Fresh noodles

In chapter 6 was concluded that non-covalent interactions have more impact on the properties of fresh noodles than covalent cross-linking reactions. Overall, all fresh noodles types required less work to fracture when containing urea, salt, or olive oil. These additions mainly interfere with respectively hydrogen bonding and ionic and hydrophobic interactions. Van Steertegem *et al*. (2013) already described that salt increases the time needed to develop wheat dough. The reduction in strength of fresh noodles containing added urea, salt, or olive oil indicates that their protein networks were less developed. These additions had a larger impact on Kieffer-rig extensibility parameters than did addition of the different model proteins, with exception of ovalbumin and S-

ovalbumin. The presence of the latter was detrimental for the extensibility and total work of fresh control noodles.

Section 6.3.3.2 described that attractive and repulsive **ionic interactions** can be estimated. At the pH of control noodles (Table 7.2), gliadin (pI *ca.* 7.8) has a net positive while glutenin (pI *ca*. 5.8) a slightly net negative charge. This suggests attractive forces between glutenin and gliadin but equally gliadin-gliadin repulsion (Section 6.3.3.2). Shielding of the ionic interactions by salt decreased all Kieffer-rig extensibility parameters (Table 7.3, symbols). At the pH of noodles containing BSA (Table 7.2), BSA and glutenin were negatively and gliadin positively charged. Addition of BSA increased the maximum force but lowered the extensibility (Table 7.1). The interactions between BSA and gliadin increased the coherence and strength of the partially developed noodle dough. Salt decreased the maximum force and total work more in noodles containing BSA than in control noodles (Tables 7.1 and 7.3). It can be concluded that ionic interactions are important in fresh control noodles and even more so in BSA containing noodles. At the pH of noodles containing soy glycinin (Table 7.2), this protein and glutenin had almost no charge while gliadin was positively charged. Even in salt solution, the net surface charge of soy glycinin is lower than that of BSA or ovalbumin (Section 5.3.1). It is expected that ionic interactions do not contribute much to the protein network in fresh soy glycinin containing noodles. At the pH of ovalbumin and S-ovalbumin containing noodles (Table 7.2), these model proteins and glutenin were negatively charged while gliadin was positively charged. The extensibility and total work was lower in (S-)ovalbumin containing than in control noodles (Table 7.1). In addition, salt use decreased the maximum force and increased the extensibility but had no significant impact on the total work for fresh ovalbumin containing noodles (Table 7.3, symbols). Thus, salt increased the flexibility and decreased the strength of the protein network in such noodles. The addition of lysozyme did not significantly change any of the Kieffer-rig extensibility parameters (Table 7.1). However, in contrast to what was the case for control noodles, all proteins in lysozyme containing noodles were positively charged suggesting mainly repulsive forces. The significant decrease of all Kieffer-rig extensibility parameters of fresh lysozyme noodles upon salt addition (Table 7.3) indicated the importance of ionic interactions in lysozyme containing noodles. With addition of salt, all Kieffer-rig extensibility parameters decreased to a larger extent in lysozyme containing than in control noodles. In contrast to gliadin, lysozyme is mainly hydrophilic at its surface and hinders protein network formation in noodles. Thus, ionic interactions contributed more to the properties of noodles containing BSA, (S-)ovalbumin and lysozyme than to those of control noodles.

Gluten proteins contain hydrophobic patches on their surface (Delcour *et al.* 2012). Addition of olive oil decreased the coherence between gluten proteins which decreased all Kieffer-rig extensibility parameters of control noodles. Of all added proteins, BSA had the highest surface hydrophobicity

(Section 5.3.1). In fresh noodles, **hydrophobic interactions** between BSA and gluten probably increased the coherence of the protein network. Inclusion of both olive oil and BSA in the recipe decreased its maximum force and total work significantly. In olive oil containing noodles, addition of BSA increased the maximum force and decreased the extensibility (Table 7.3). It is hypothesized that hydrophobic patches in both BSA and gluten can interact with triacylglycerols and each other thereby increasing the coherence of the protein network but reducing its flexibility. In contrast, ovalbumin or lysozyme decreased the maximum force without altering the extensibility in olive oil containing noodles (Table 7.3). They both reduced the strength but not the flexibility of the protein network in noodles. It is suggested that the added olive oil mainly interacts with gluten as unheated soy glycinin, (S-)ovalbumin and lysozyme are mostly hydrophilic at their surface. In noodles containing ovalbumin, olive oil use decreased the maximum force and increased the extensibility. Higher concentrations of olive oil (*ca.* 12% on dm noodles) in control noodles had similar impact on these parameters (Section 6.3.3.3). In noodles containing lysozyme, only the maximum force and total work significantly decreased as a result of olive oil use. It is suggested that hydrophobic interactions in fresh noodles mainly occur between gluten and BSA.

**Hydrogen bonds** are important for the extension properties of wheat dough (Belton 1999). The presence of urea in control noodles was more detrimental for the Kieffer-rig extensibility parameters than that of salt or olive oil. For ovalbumin containing noodles, urea was the only additive which reduced the total work significantly. The reduction in maximum force and total work was higher for noodles containing BSA than for control noodles. However, when urea was included in the recipe, the differences between noodles with various model proteins were relatively low. About two thirds of the protein in these noodles is wheat protein. To conclude, hydrogen bonds are of main importance in the gluten network irrespective of the noodles type studied.

Neither the model proteins nor chemical additives positively impacted noodle extensibility and total work of fresh control noodles. Impacting the ionic, hydrophobic interactions or the hydrogen bonds between proteins in control and BSA containing noodles largely decreased the coherence and strength of noodle dough. Soy glycinin impacted noodle dough mainly through hydrogen bonding as it is hardly charged and contains little hydrophobic groups (Section 5.3.1). Ovalbumin and lysozyme containing noodles were mostly affected by inclusion of salt and urea in the recipe. Salt increased the extensibility of ovalbumin containing noodles which otherwise had a more rigid protein network. Shielding the positive charges in lysozyme containing noodles decreased the flexibility of the protein network in fresh noodles. Thus, ovalbumin, S-ovalbumin and lysozyme mainly interacted by ionic and hydrogen interactions in noodle dough.

### 7.4.2 Optimally cooked noodles

During noodle cooking, water advances to the core. Starch swells and proteins polymerize following first-order kinetics. Noodles containing (S-)ovalbumin, which polymerized fast (high k value, Figure 7.1) during the first minutes of cooking, had long optimal cooking times while noodles containing soy glycinin or lysozyme, which polymerized slowly (low k value) during cooking, had short optimal cooking times. In agreement with Section 6.3.3 the optimal cooking time seemed positively correlated with the order of rate loss of protein extractability in SDS medium. The abundance of free SH groups clearly impacted the rate of protein network formation and the incorporation of α- and γ-gliadins therein. Nevertheless, the rapidly developed protein network of S-ovalbumin containing noodles could not withstand starch swelling, resulting in high water absorption and cooking loss. Lysozyme or soy glycinin containing noodles polymerized slowly due to the lack of free SH groups to initiate SH-SS exchange reactions in these added proteins. Also, the repulsive ionic interactions between all proteins in noodles containing lysozyme probably disfavored polymerization. Furthermore, as a result of differences in their MW, more interconnections were required to create a continuous protein network in noodles containing lysozyme (*ca.* 14 kDa) than in those containing soy glycinin (*ca.* 360 kDa). Although the protein network of lysozyme containing noodles was faster and more extensively developed than that of soy glycinin containing noodles, the optimal cooking time of the former was lower and more protein leached into the cooking water.

In Chapter 6, it was suggested that covalent cross-links and hydrogen bonds are the main determinants of the quality of optimally cooked noodles. The fast and highly developed covalent protein network in (S-)ovalbumin containing noodles fixed their structure resulting in a lower extensibility than for the other noodle types. Inclusion of BSA, soy glycinin or lysozyme in the noodle recipe increased the maximum force and total work after optimal cooking. However, at the optimal cooking time, the covalent protein network of lysozyme containing noodles was the least developed amongst all noodle types. The possible impact of non-covalent interactions on the strength of cooked noodles is outlined below.

The above results suggest that an optimum extent of covalent network formation is necessary if one is to obtain high quality noodles. On the one hand, fast polymerization of proteins as noted for (S-)ovalbumin containing noodles impairs the extensibility and cooking quality of optimally cooked noodles. It tightens the protein network making it less flexible to withstand starch swelling during cooking and the Kieffer-rig protocol after cooking. On the other hand, more protein leached into the cooking water when insufficient protein was incorporated in the protein network. This investigation opens perspectives of using naturally occurring proteins to enhance not only the nutritional value but

also the quality of noodles and pasta. Indeed, for pasta, Bruneel *et al.* (2016) hypothesized likewise that an optimum extent of gliadin incorporation is critical to obtain high quality cooked pasta after having included redox agents, oxidants and N-ethylmaleimide in the recipe and evaluated their impact on pasta quality.

### 7.4.3 Overcooked noodles

After 20 min cooking, the SDS-EP loss had reached a plateau for all noodles types (Figure 7.1). However, the impact of globular proteins on the rate of polymerization and the final SDS-EP plateau, as well as on non-covalent interactions could be linked to differences in properties between various noodle types after prolonged cooking.

Urea was the only additive which weakened the total work of overcooked control noodles (Table 7.3, symbols). It reduced the extensibility of noodles while the maximum force remained the same. These data point to the importance of hydrogen bonds during extension of gluten in fresh and cooked noodles. Hydrogen bonds in gluten are mainly formed during cooling (Lagrain *et al.* 2010). The use of both model proteins and urea in noodle recipes did not significantly impact the total work of overcooked noodles (Table 7.3). Still, addition of BSA or ovalbumin to urea containing noodles increased the maximum force probably due to increased SS cross-linking (Table 7.3, letters). Furthermore, protons with highest mobility determined by $^{1}$H-NMR were more strongly bound in overcooked lysozyme containing noodles than in control noodles, which suggested the importance of hydrogen bonding in these noodles. To summarize, addition of urea to lysozyme containing noodles decreased all Kieffer-rig extensibility parameters, as also noted for BSA but not for ovalbumin containing noodles.

Addition of salt did not impact the Kieffer-rig extensibility parameters of overcooked control or ovalbumin containing noodles but decreased those of overcooked noodles containing BSA or lysozyme (Table 7.3, symbols). Shielding of ionic interactions by salt negatively impacted protein network formation in fresh BSA or lysozyme containing noodles and had an even more pronounced impact on these noodles when overcooked. Salt not only impacts ionic interactions but also reduces protein polymerization in noodles (Rombouts *et al.* 2014). The covalent protein network of noodles containing both salt and BSA or lysozyme were probably less developed than those in their unsalted counterparts. The impact of salt on the covalent network of ovalbumin was not sufficient to reduce its extensively developed protein network and to increase its Kieffer-rig extensibility properties.

Addition of olive oil did not impact the Kieffer-rig extensibility parameters of overcooked control and BSA containing noodles but increased or decreased those of ovalbumin or lysozyme containing

noodles, respectively (Table 7.3, symbols). Olive oil decreases the rate of polymerization and the extent of interconnections between proteins during cooking (Section 6.3.3.3). Ovalbumin and lysozyme contain a hydrophobic core which becomes exposed upon their denaturation. Most likely, olive oil affects the polymerization of these denatured proteins. For ovalbumin containing noodles, the negative impact of olive oil on protein polymerization improved the flexibility of the protein network resulting in increased Kieffer-rig extensibility parameters. The inferior protein network of lysozyme was negatively impacted by the presence of olive oil. It decreased Kieffer-rig extensibility parameters. Furthermore, non-covalently bound lysozyme molecules can refold during cooling which increases the amount of hydrophilic residues at its surface (Wu *et al.* 2015).

In noodles containing (S-)ovalbumin, a strong covalent protein network was very rapidly formed due to the high level of accessible free SH groups. As a consequence, the protein network formed could not withstand starch swelling. Optimally cooked and overcooked (S-)ovalbumin containing noodles were less extensible than control noodles. Only the addition of BSA or soy glycinin positively impacted maximum force and total work of overcooked noodles. These noodle types already had an optimum network after optimally cooking and continued having the best quality during prolonged cooking. The hypothesis of Chapter 6 that covalent cross-links and hydrogen bonds are the main determinants of Kieffer-rig extensibility parameters in cooked noodles can now be refined. Hydrophobic and ionic interactions can also impact noodle quality but probably indirect by hindering covalent network formation.

## 7.5 Conclusion

In all fresh noodle types studied, both ionic and hydrophobic interactions and hydrogen bonds contribute to the coherence and strength of noodle dough. This was demonstrated by the quality loss in most noodle types as a result of salt, olive oil or urea addition. The properties of fresh noodles are largely impacted by hydrogen bonds. BSA, (S-)ovalbumin or lysozyme addition increase the contribution of ionic interactions in fresh noodles. BSA and gluten proteins strengthen the noodle structure by hydrophobic interactions. Thus, the characteristics of the model proteins included in the recipe impact the properties of fresh noodles.

Introducing free SH groups by inclusion of model proteins containing such residues in the recipe increases the rate and extent of gliadin incorporation in the protein network during cooking. This is *e.g.* the case in BSA or (S-)ovalbumin containing noodles. Excessive protein polymerization decreases the extensibility and cooking quality of (S-)ovalbumin noodles, probably because the protein network is not flexible enough to cope with starch swelling during cooking. Addition of lysozyme, a small protein with no free SH groups, lowers the rate and extent of protein polymerization and results in

low cooking quality. An optimum balance between the rate and extent of covalent network formation and starch gelatinization is necessary to obtain high quality noodles. Besides covalent cross-links, hydrogen bonds determine Kieffer-rig extensibility parameters of cooked noodles. In addition, hydrophobic and ionic interactions may play an important role in cooked noodles because they indirectly affect covalent network formation.

Proteins from different sources can not only enhance the nutritional value of wheat-based noodles but also noodle quality. Insights in this Chapter can be helpful for selecting protein sources as egg substitutes in egg noodles. While none of the model proteins tested here have a clear positive impact on the properties of fresh noodles, superior Kieffer-rig extensibility parameters are noted for optimally cooked noodles when BSA or soy glycinin are part of the recipe. In addition, these noodle types are less affected by overcooking. A general conclusion is that an optimal extent and timing of covalent protein network is essential if one is to obtain high quality noodles. Moreover, non-covalent interactions contribute to the overall quality of wheat-based noodles during and after cooking.

Lysozyme containing dough is easy to handle and produces both fresh and optimally cooked noodles with Kieffer-rig extensibility parameters comparable or superior to those of control noodles. Furthermore, their low optimal cooking time is convenient. However, due to insufficient SS cross-links, more protein leaches during cooking and these noodles are also less resistant to overcooking than BSA or soy glycinin containing noodles. Use of the latter two proteins does not result in optimal dough properties, but still increases the strength of optimally cooked noodles. These results suggest that the inclusion of a mixture of lysozyme on the one hand and BSA or soy glycinin on the other hand can enhance dough processability, Kieffer-rig extensibility properties, as well as cooking quality. Further experiments are needed to investigate this hypothesis.

# General conclusions and perspectives

Interactions and reactions between proteins of different sources can induce co-protein effects which impact the properties of food systems. Before this work was executed, the relation between protein structure and functionality, especially in mixtures of different protein types was insufficiently known. This dissertation studied interactions and reactions between wheat gluten and egg, soy and whey with wheat proteins in model systems under various conditions and in wheat-based noodles.

To study heat-induced covalent cross-linking of and between different types of food proteins, a size exclusion chromatography method was optimized with SDS medium both as eluent and extraction medium. Size exclusion chromatography separates proteins based on their hydrodynamic volume in a given extraction medium and is a powerful tool for evaluating changes in molecular weight distributions. However, co-solvents in extraction media and elution solvents can induce non-size effects which, if not well understood, can lead to misinterpretation of the chromatograms. With ACN medium as eluent, co-solvents impact the separation of albumins and globulins. The presence of salts or SDS in the extraction medium increases while urea decreases the occurrence of non-size effects. With SDS medium as eluent, no remarkable non-size effects occur in the presence of various co-solvents.

Due to differences in hydrophilicity, some proteins are completely soluble in water while others are better soluble in a more hydrophobic environment such as aqueous ethanol. It was demonstrated that the colloidal stability, the conformation and the availability of reactive groups of proteins can

differ in water from that in aqueous ethanol. The presence of aqueous ethanol decreases the rate of β-elimination, SH oxidation and SH-SS exchange reactions during heating.

Also, different protein types influence each other's polymerization, even when they are phase-separated. In water but not in aqueous ethanol, gliadin-BSA and gliadin-soy glycinin mixtures polymerize to a larger extent than expected based on the polymerization of the isolated proteins during heating at 100 °C. This enhanced polymerization in mixtures of proteins is referred to as a positive co-protein effect. Thermodynamic compatibility is not the key parameter impacting polymerization between different proteins. This opens perspectives to co-protein effects between globular proteins and wheat gluten in water, the most commonly used solvent in food applications.

The presence and accessibility of free SH groups and SS bonds are key parameters during heat-induced polymerization of globular proteins in water at 100 °C. Accessible free SH groups initiate cross-linking mainly through SH oxidation and SH-SS exchange reactions. Small amounts of intramolecular SS bonds limit the formation of a continuous covalent protein network. In egg white, proteins polymerize more than expected based on observations for the isolated proteins. Gliadin polymerizes faster in the presence than in the absence of glutenin. Nevertheless, polymerization of whole egg proteins is the sum of that of egg white and egg yolk proteins. In mixtures with gluten, negative co-protein effects are noted for lysozyme, no co-protein effects for soy glycinin and egg yolk, and positive co-protein effects for BSA, (S-)ovalbumin, egg white, whole egg, wheat albumin, wheat globulin and defatted egg yolk. Different protein characteristics of globular proteins [*i.e.* monomeric particle size, free SH content, cysteine level, denaturation temperature of the largest fraction, ζ-potential and surface hydrophobicity from the unheated and heated (1 min; 100 °C) samples] are related to their co-protein effect with gluten. The level of accessible free SH groups and the surface hydrophobicity of unfolded proteins are the main protein characteristics determining co-protein effects in mixtures with gluten during heating (100 °C) in water. (S-)Ovalbumin (containing four free SH groups) enhances polymerization of gluten, especially that of gliadin, more than BSA (containing one free SH group). These novel fundamental insights into protein cross-linking of complex protein mixtures are important in the light of the growing demand for sustainable alternatives for globular animal proteins. Predicting the effect of food proteins on each other's covalent network formation can provide a basis for developing new food formulations.

The co-protein effects between globular and gluten proteins in model systems can be linked to the rate and extent of covalent network formation in noodles (Figure G1). Globular proteins like (S-)ovalbumin with positive co-protein effects in model systems with gluten result in poor noodle quality [high optimal cooking times, low cooking quality (high water absorption and cooking loss) and

low Kieffer-rig extensibility parameters]. The covalent protein network formed very fast during cooking of these noodle types and cannot cope with starch swelling. Also, noodles in which insufficient protein polymerization (*e.g.* lysozyme or egg yolk containing noodles) occurs result in low cooking quality and/or Kieffer-rig extensibility properties. Lysozyme and egg yolk proteins decrease or do not impact the rate and/or extent of covalent network formation in model systems with gluten and in wheat-based noodles. Superior noodle quality depends on the balance between the timing and extent of protein network formation and starch gelatinization. Noodles containing whole egg have high Kieffer-rig extensibility properties and noodles containing BSA or soy glycinin are least affected by overcooking.

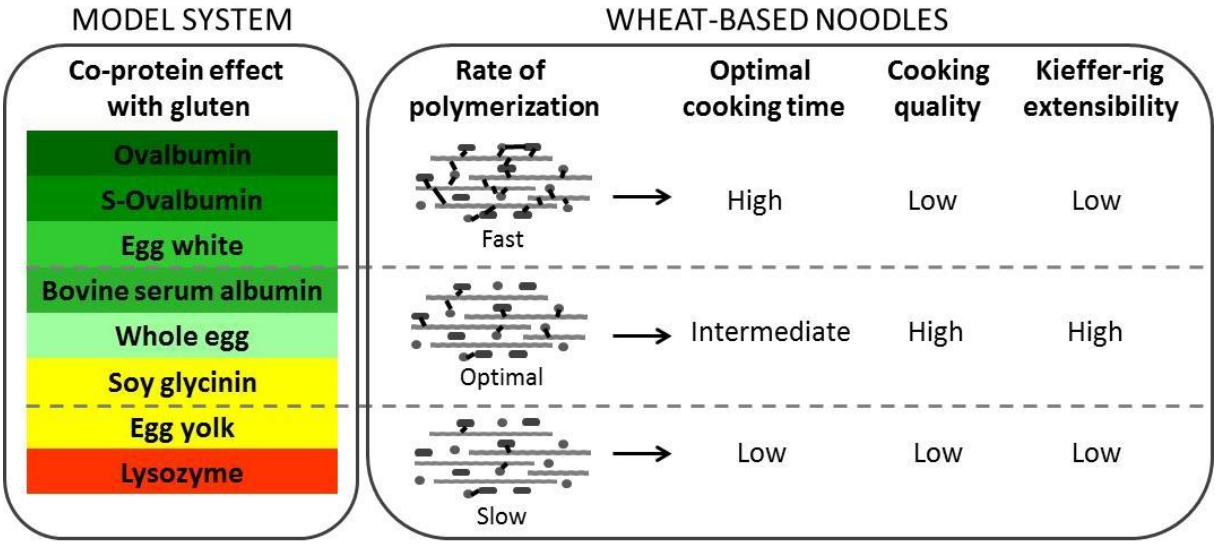


**Figure G1: Schematic overview of the relation between co-protein effects of globular and wheat proteins in model systems and noodles after cooking. The colored bars indicate positive (green, dark > light), none (yellow) or negative (orange) co-protein effects in model systems.**

Non-covalent interactions are important for the coherence and strength of noodle dough. The characteristics of added proteins impact the properties of fresh noodles. Introducing free SH groups in the form of added proteins increases the rate and extent of gliadin incorporation in the protein network during cooking of such noodles. Covalent cross-links and hydrogen bonds mainly determine the Kieffer-rig extensibility parameters of cooked noodles. Hydrophobic and ionic interactions impact covalent network formation during cooking and may indirectly impact noodle quality. Thus, non-covalent interactions and covalent cross-links contribute to the overall quality of wheat-based noodles during and after cooking.

This work showed that different protein (sources) can enhance not only the nutritional value but also the structural properties of noodles. Inclusion of some egg, soy, and whey proteins in noodle recipes can lead to superior noodle strength and/or cooking quality. Overall, globular proteins impact the rate and extent of protein network formation in a similar way both in model and in noodle systems.

However, for noodles it is crucial to take into account the fine balance between protein polymerization and starch gelatinization. Thus, the highest co-protein effects do not necessarily lead to superior noodle quality.

The obtained model which relates protein characteristics of globular proteins with co-protein effects in the presence of gluten can form a basis for developing cereal-based food products. Depending on the required timing and extent of protein polymerization, proteins with specific characteristics can be selected to enhance the properties of certain cereal-based food products. As such, egg proteins can be (partially) replaced by more sustainable and cost-effective protein sources.

While this work has increased insight in the impact of different types of proteins on each other's denaturation, aggregation and polymerization, some research needs still exist. This study focused on heat treatment of different protein types above their denaturation temperature. Most likely, two proteins influence each other's polymerization differently when only one of both proteins is denatured than when both proteins are denatured. More knowledge would further support the development of innovative wheat-based food system applications. For instance, the impact of structural modifications of globular proteins on their functionality should be investigated. Gels of isolated globular proteins can be strengthened by structuring them into fibrils (Akkermans *et al.* 2008; Jung *et al.* 2010). The impact of egg, whey or soy fibrils on protein network formation in and quality of wheat-based products is unknown. Furthermore, a better understanding of co-protein effects between globular proteins and proteins in other cereals such as maize, rice and oats can open perspectives for the gluten-free industry. Proteins in gluten-free cereals do not form a visco-elastic network. Therefore, other non-cereal proteins are often necessary to increase the coherence of the protein network and to enhance the quality of gluten-free products. The link between protein characteristics and protein functionality in mixtures of cereals would support the development of cost-effective formulations and superior end-product quality. Finally, the functionality of other protein sources can be explored in the context of egg replacement in different food products.

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# List of publications

## Publications in international peer reviewed journals

Pauly, A., Pareyt, B., Lambrecht, M.A., Fierens, E. and Delcour, J.A. **2013**. Flour from wheat cultivars of varying hardness produces semi-sweet biscuits with varying textural and structural properties. LWT – Food Science and Technology 53: 452-457.

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Lambrecht M.A., Rombouts I., Nivelle M.A., Delcour J.A. The role of wheat and egg constituents in the formation of a covalent and non-covalent protein network in fresh and cooked egg noodles. Journal of Food Science, *accepted for publication*.

## Contributions at international meetings published as abstract

Lambrecht M.A., Rombouts I., De Ketelaere B. and Delcour J.A. **2016**. Heat-induced polymerization of different non-wheat food proteins and mixtures thereof with wheat gluten. Food Chemistry Conference, Amsterdam, The Netherlands.

Rombouts I., Lambrecht M.A., Lagrain B., Scherf K.A., Carpentier S.C., Koehler P. and Delcour J.A. **2016**. Study of posttranslational modifications in proteins during food processing using tandem mass spectrometry with collision-induced, electron-transfer and higher-energy collisional dissociation. Food Chemistry Conference, Amsterdam, The Netherlands.

Lambrecht M.A., Rombouts I. and Delcour J.A. **2015**. Walter Bushuk Graduate Research Award in Cereal Protein Chemistry - Cross-linking of gluten, globular proteins and mixtures thereof in aqueous ethanol and water. AACCI Annual Meeting, Minneapolis, MN, USA.

Lambrecht M.A., Rombouts I. and Delcour J.A. **2015**. Impact of eggs and egg fractions on quality of fresh egg noodles. C&E spring meeting, Budapest.

Lambrecht M.A., Rombouts I. and Delcour J.A. **2015**. Covalent network formation during heating of food proteins in aqueous ethanol and water. C&E spring meeting, Budapest.

Lambrecht M.A., Rombouts I. and Delcour J.A. **2014**. Impact of extraction and elution media on separation of proteins by size exclusion high performance liquid chromatography. 13th European European Young Cereal Scientists and Technologists Workshop, Freising-Weihenstephan, Germany.

Pauly A., Pareyt B., Lambrecht M.A., Fierens E. and Delcour J. **2013**. Impact of puroindolines on semi-sweet biscuit quality. C&E Spring Meeting, Leuven, Belgium.

Pauly A., Pareyt B., Lambrecht M., Fierens E. and Delcour J. **2012**. Flour from wheat cultivars of varying hardness affects textural and structural properties of semi-sweet biscuits. AACC International Annual Meeting, Hollywood, FL, USA.